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## PREPARATION AND COMPARISON OF STANDARDS FOR THE ESTIMATION OF CREATINE AND CREATININE.

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Since the almost universal adoption of the Folin colorimetric method for the estimation of creatine and creatinine, various substances have been proposed as standards with which the unknown solution is to be compared. Folin originally (1) proposed 0.5 N potassium bichromate as a standard; later (2) he advocated creatinine zinc chloride; Thompson (3) proposed creatinine picrate and creatinine potassium picrate; pure creatinine has also been employed. Each of these standards possesses certain advantages and disadvantages.

A substance to be used as a standard for analysis should have, if possible, certain characteristics. It should be of definite composition, easily purified, sufficiently stable to permit its being thoroughly dried without decomposition, and it should present some simple test by which its purity may be ascertained. In addition, it is desirable that it shall be easily prepared and not too costly. Heretofore the difficulty and expense involved in the preparation of pure creatinine compounds have limited somewhat their use as standards. In view of the fact that reasonably pure creatine is now available (4) in the market at much reduced prices, it seems desirable to consider the standards indicated above with reference to their preparation from commercial creatine.

*Potassium Bichromate.*—While potassium bichromate is widely used because of its permanence, convenience, and inexpensiveness, it may be dismissed at once as a really satisfactory standard for general use, as its color is not an exact duplicate of that developed by the Folin reaction in solutions of creatinine. This means that to obtain concordant results a most exact procedure is necessary, and only certain types of colorimeter can be used, as

## 2      Estimation of Creatine and Creatinine

a potassium bichromate solution which will match at one depth a creatinine solution prepared for analysis, will not match it at another depth. It is true that by the use of a varying factor (5) comparisons may be made at different depths, but this complicates the calculation, and at best the color match is not perfect. Furthermore, the color developed in the reaction is somewhat sensitive to conditions of temperature, time, concentration, etc., and it is, therefore, much preferable to carry out the analysis with the unknown solution and a standard containing a known quantity of creatinine, the two being treated similarly in all respects.

*Creatinine Zinc Chloride.*—Creatinine zinc chloride proposed by Folin (2) as a standard has much to recommend it. It is a very characteristic compound of creatinine, relatively insoluble, and can be used to separate creatinine from various impurities.

Up to the present, methods for its preparation from creatine and its purification have not been altogether simple. A new reaction, however, described below, permits a very rapid and simple method for preparing a pure product. The substance possesses no definite simple test of purity, and reliance must be had upon recrystallization.

*Creatinine Picrate.*—Creatinine picrate proposed as a standard by Thompson (3) possesses many advantages. It can be prepared very simply, as shown below, from commercial creatine. It is readily purified by crystallization from hot water, being relatively insoluble in cold water, and having a high temperature coefficient of solubility. It possesses a definite melting point (205°C.) by which the purity of a given sample may be tested. A disadvantage is that the solubility in cold water limits the preparation of standard solutions to concentrations of 0.5 mg. of creatinine per cubic centimeter, but as will be shown below, this is quite satisfactory for standard methods of analysis.

*Creatinine.*—Creatinine, itself, is unsatisfactory as a standard because of the difficulty involved in its preparation and the inevitable uncertainty as to its purity. Experiments carried out by the writer confirm the conclusions of Thompson (3) that in recrystallizing creatinine from water or aqueous alcohol, some hydration to form creatine may take place. Thus, a sample prepared by the method of Folin and Denis was found after three recrystalliza-

tions from aqueous alcohol to contain several per cent of creatine, apparently partially formed during the purification. Since creatinine does not possess a definite melting point its purity cannot be simply tested. This does not mean, of course, that pure creatinine cannot be prepared (6), but uncertainty as to the presence of creatine in any given sample, and the difficulty of removing it if present, make creatinine unsatisfactory as a basic standard.

#### EXPERIMENTAL.

In order to obtain data upon which to base a comparison of creatinine zinc chloride and creatinine picrate, from the standpoint of ease of preparation from creatine, purity, and other properties, an experimental study was made, the results of which are briefly summarized below.

All the standard methods for the preparation of creatinine zinc chloride were abandoned when it was discovered that solid creatine reacts readily with anhydrous zinc chloride, on heating, to give creatinine zinc chloride, the anhydrous zinc chloride acting first as dehydrating agent to form creatinine, and then reacting to form the salt. The reaction apparently occurs under a wide range of conditions, but the following technique was finally adopted as giving the best yield and purest product.

Commercial creatine is ground in a mortar with an equal weight of anhydrous zinc chloride. The mixture is then placed in a beaker, dish, or casserole, and is heated over a small flame or sand bath, with constant stirring. As the temperature is raised the mixture begins to melt and around 120–130°C. (depending somewhat on the rate of heating and the moisture which may have been absorbed) becomes a viscous mass from which bubbles of water vapor are given off. Within a few minutes the mass suddenly solidifies to a perfectly dry residue consisting of creatinine zinc chloride (containing, of course, the excess zinc chloride). The whole reaction is complete within about 5 minutes after beginning to raise the temperature. If crude creatinine zinc chloride is desired it is only necessary to leach the mass with a little cold water or aqueous alcohol to dissolve out the excess zinc chloride. If a pure product is desired the residue may be dissolved directly (following Folin) in about ten times its weight

#### 4      Estimation of Creatine and Creatinine

of boiling 25 per cent acetic acid, and 2 volumes of alcohol are added to the mixture. On cooling crystalline creatinine zinc chloride separates in practically quantitative yield (based on the original creatine), and of a purity comparable with the best product obtained by repeated crystallization.

Creatinine picrate was prepared from commercial creatine, by converting the latter into creatinine hydrochloride, which can be simply effected by dissolving creatine in an excess of 6 N (or other concentration) hydrochloric acid, and evaporating to dryness on a water bath (Benedict, 7). Other methods, such as prolonged boiling with acid, can also be employed, and doubtless other acids, but this procedure seemed the simplest possible. The creatinine hydrochloride was then dissolved in a little water and added to a slight excess of pure picric acid previously dissolved in either boiling water or boiling alcohol. After cooling the mixture, the precipitated creatinine picrate was filtered off, washed with a little cold water, recrystallized once from boiling water, washed with alcohol, and dried at 100°C. All samples thus prepared melted within 0.5° of 205°C. (uncorrected) (most texts give 213°, but Thompson reports 205°), and further tests showed them apparently perfectly pure.

Standard solutions were then prepared from creatinine zinc chloride and creatinine picrate prepared as described above, and also from creatinine zinc chloride prepared from pure creatinine and repeatedly recrystallized.

The creatinine zinc chloride was prepared to contain 1 mg. of creatinine per cubic centimeter (1.602 gm. of salt per liter). The creatinine picrate was not sufficiently soluble for the concentration to be used, and, therefore, it was made to contain 0.5 mg. of creatinine per cubic centimeter (1.5119 gm. of salt per liter). This is a nearly saturated solution at room temperature. The standard solutions were then treated with picric acid and alkali as in the Folin method. Investigators have disagreed as to the best procedure for this purpose, but it seems agreed that in any case an identical procedure should be followed with standard and unknown, and that approximately the same amount of creatinine should be present in both. A procedure was adopted, therefore, which is essentially that employed by both Folin and Thompson. 10 mg. of creatinine (10 cc. of the creatinine zinc chloride solu-

tion, 20 cc. of the creatinine picrate solution) were treated with 25 cc. of saturated picric acid solution and 10 cc. of 10 per cent NaOH. The mixtures were allowed to stand for 7 minutes and were then diluted to 500 cc. with water, and were compared in various colorimeters.

The instruments used were a Sargent, a Bock-Benedict, and a Duboscq colorimeter, and in them the solutions were matched in depths varying from 10 to 100 mm. In every case the readings with the three standards agreed exactly within the experimental error of matching. In order to show that approximate results may be obtained in creatinine estimation without any colorimeter, the two solutions were also compared in Nessler tubes, and it was found that very fair results were obtained. One great advantage of a standard creatinine solution is that no particular depth of reading is necessary, and the concentration of creatinine in the unknown solution is given directly by the relation,

$$\frac{\text{Concentration of unknown}}{\text{Concentration of standard}} = \frac{\text{Depth of standard}}{\text{Depth of unknown}}$$

#### DISCUSSION.

The data outlined above, together with considerable general experience with the standards in question, seem to justify the following conclusions.

1. Both creatinine zinc chloride and creatinine picrate may be readily prepared from commercial creatine, in a state of sufficient purity for them to be utilized as standards for analysis by the Folin method.
2. Creatinine picrate has the advantage of possessing a simple test of purity (melting point), and the disadvantage for some purposes of a limited solubility.
3. Creatinine picrate, both solid and in solution, is apparently perfectly stable over considerable intervals of time. Creatinine zinc chloride solutions, like pure creatinine solutions, slowly change in concentration due to partial creatine formation, but this can be readily overcome by adding acid to the solution. (Folin has suggested that the creatinine zinc chloride should be dissolved in 0.1 N HCl instead of water.)
4. Many metallic picrates are quite explosive, both by shock and by heat. In the writer's experience this is not true of creati-

## 6        Estimation of Creatine and Creatinine

nine picrate. It can be heated above its melting point with only gradual decomposition, and efforts to explode it by ordinary shock have failed.

5. The selection of creatinine picrate or creatinine zinc chloride as a standard is largely a matter of choice, but the advantages of either over other standards, in view of the fact that any colorimeter, any depth of layer, and many concentrations of standard solution, may be employed, would appear to justify their more universal adoption, particularly when they may be so simply prepared.

### SUMMARY.

A comparison has been made of the merits of different standards which have been proposed for creatinine determination.

A new and simple method has been devised for the preparation of creatinine zinc chloride. The advantages of creatinine zinc chloride and creatinine picrate as standards have been emphasized.

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## LIPASE STUDIES.

### II. A COMPARISON OF THE HYDROLYSIS OF THE ESTERS OF THE DICARBOXYLIC ACIDS BY THE LIPASE OF THE LIVER.

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In a previous paper (1) a study of the hydrolysis of the diethyl esters of malonic and succinic acids by the lipase of hog liver was reported. On the basis of the acidity developed when a glycerol extract of hog liver was allowed to act upon these esters, it was considered that the reaction proceeded to an equilibrium which corresponded to the removal of one ethyl group from the diethyl esters and that the further hydrolysis of the monoethyl ester did not take place readily, if at all.

It seemed desirable to extend this study to the higher members of this homologous series of dicarboxylic acids in order to determine whether this type of hydrolysis was common to all members of the series. Accordingly, the hydrolysis by lipase of the diethyl esters of the homologous acids, glutaric and adipic, has been studied.

#### EXPERIMENTAL.

*Preparation of Lipase.*—The lipase was prepared from fresh hog liver by extraction with glycerol, according to the method of Kanitz (2), the extract being prepared within 2 hours after the slaughter of the animal. After a thorough admixture the extraction mass was allowed to stand filtered through cheese-cloth as needed. The extract retained its lipolytic activity unimpaired for several months.

*The Esters.*—The ethyl propionate, and diethyl malonate and succinate were commercial products. Diethyl glutarate was especially prepared for this work by Dr. C. S. Marvel of the

Organic Division. Adipic acid was prepared from cyclohexane by oxidation with nitric acid, and esterified in the laboratory in which this research was conducted. The purity of the esters was determined by means of saponification values. Inasmuch as it was difficult to weigh the liquid esters exactly, solutions of normality, approximating that desired, were prepared and their exact value in terms of sodium hydroxide as required for saponification was determined. In order to have the esters completely in solution, low concentrations (0.05 to 0.0125 N) of ester were employed.

*Determination of the Action of the Lipase on the Ester.*—0.5 cc. portions of the glycerol extract were added to 25 cc. of the standard ester solution, the flasks were incubated at room temperature for varying periods of time, and the acidity developed in the reaction was then titrated with standard sodium hydroxide, with phenolphthalein as an indicator. The flasks were arranged in pairs and one pair was titrated after 30 minutes, a second pair after 1 hour, etc. Each pair of the flasks was also retitrated at the intervals shown in the tables and the figures given represent the total volume of standard alkali required for neutralization. The figures presented in the tables are the averages of check determinations from which the blanks due to the acidity of the extract and of the esters have been subtracted.

In all the tables the last figure in each vertical column represents the amount of alkali required for neutralization of the acidity developed during the period represented. Each figure in the horizontal column to the right of the first figure represents this amount of alkali plus the additional amounts of alkali required for retitration at the intervals indicated. Thus in Table I under the heading "2 hours," the figure 4.27 represents the average acidity developed in two flasks which were first titrated after 2 hours incubation; 4.70 represents the average total acidity of a pair of flasks, which were titrated at the end of an hour and again 1 hour later after a total period of incubation of 2 hours, etc.

#### DISCUSSION.

*Ethyl Propionate.*—In order to afford a control on the hydrolysis of a simple ester, the results of a typical experiment with ethyl propionate are presented in Table I. The data show that under

the conditions of the experiment, 0.025 N ethyl propionate was hydrolyzed to the extent of 85 to 90 per cent before equilibrium was reached.

*Diethyl Malonate and Diethyl Succinate.*—The results of the experiments with the diethyl esters of malonic and succinic acids (Table II) were in harmony with the results of Christman and Lewis (1) previously reported. They show that hydrolysis tended to proceed rapidly to a point of equilibrium corresponding to removal of one ethyl group and after this point was reached, further action was very slow.

TABLE I.  
*Hydrolysis of 0.025 N Ethyl Propionate by Lipase.*

25 cc. portions of ester were used. For the complete saponification of this amount of ester 5.8 cc. of 0.09872 N NaOH were required.

Time...30 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	10 hrs.	11 hrs.
Standard NaOH required for neutralization of acidity.								
cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
3.05	4.70	5.05	5.07	5.07	5.07	5.07	5.07	5.07
	3.72	4.70	4.80	4.80	4.80	4.80	4.80	4.80
		4.27	4.45	4.55	4.55	4.55	4.55	4.55
			4.35	4.55	4.55	4.55	4.55	4.55
				4.92	5.17	5.17	5.17	5.17
					4.97	5.02	5.02	5.02
						5.05	5.05	5.05
							5.00	5.00
								5.15

*Diethyl Glutarate (Table III).*—When diethyl glutarate in dilute solution was acted on by liver lipase under the standard experimental conditions, the course of the hydrolysis was quite different from that observed with the diethyl esters previously studied. Thus with a 0.05 N solution of the ester, the reaction progressed until at the end of 7 and 9 hours, respectively, approximately 75 and 78 per cent of the ester had been split. With a 0.025 N solution hydrolysis was practically complete at the end of 5 hours. These values were obtained with flasks in which the acidity developed was neutralized only at the end of the stated period of time.

## Lipase. II

If, however, the acidity developed was neutralized at frequent intervals, the results were quite different. If the acidity developed corresponded to a cleavage of 50 per cent or more of the ester, neutralization of this acidity checked further hydrolysis. Thus, with 0.05 N ester at the end of 30 minutes, an acidity of 3.30 cc.,

TABLE II.

*Hydrolysis of 0.05 N Solutions of Diethyl Malonate and Succinate by Lipase.*

25 cc. portions of ester were used. For the complete saponification of this amount of these esters 12.6 and 12.2 cc., respectively, of 0.09872 N NaOH were used.

Diethyl malonate.							
Time....30 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	7 hrs.	9 hrs.
Standard NaOH required for neutralization of acidity.							
cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
2.00	3.62	5.52	6.00	6.25	6.30	6.40	6.40
	3.80	5.57	6.07	6.22	6.25	6.25	6.30
		4.75	5.75	6.10	6.25	6.25	6.30
			5.20	5.82	6.12	6.12	6.12
				5.55	6.02	6.35	6.40
					5.67	6.45	6.52
						6.00	6.35
							6.10
							6.10

Diethyl succinate.							
2.67	5.15	5.70	5.72	5.72	5.72	5.75	5.75
	4.32	5.97	6.02	6.02	6.02	6.07	6.07
		5.70	6.02	6.02	6.02	6.05	6.05
			5.90	6.05	6.05	6.05	6.05
				6.07	6.10	6.10	6.10
					6.05	6.15	6.15
						6.15	6.15
							6.20

corresponding to a hydrolysis of approximately 27 per cent of the ester, was neutralized. In the next 30 minute period further acidity developed so that the total alkali required for neutralization was 5.62 cc., equivalent to a cleavage of approximately 50 per cent of the ester. Further hydrolysis of this set of flasks was checked, only 0.18 cc. of normal alkali being required to

titrate the acidity developed in the following 8 hours. Similarly in the flasks first titrated at the end of an hour, an acidity of 5.20 cc. was developed; *i.e.*, a 44 per cent hydrolysis had occurred. During the next hour a small amount of cleavage took place which corresponded to a total hydrolysis of 50 per cent of the ester. No further activity occurred during the subsequent hours. Simi-

TABLE III.

*Hydrolysis of 0.05 and 0.025 N Solutions of Diethyl Glutarate by Lipase.*

25 cc. portions of the ester were used. For complete saponification of this amount of these esters 11.8 and 5.8 cc. of 0.09872 N NaOH, respectively, were required.

0.05 N diethyl glutarate.							
Time....30 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	7 hrs.	9 hrs.
Standard NaOH required for neutralization of acidity.							
cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
3.30	5.62	5.75	5.75	5.75	5.75	5.80	5.80
	5.20	5.97	5.97	5.97	5.97	5.97	6.05
		6.32	6.52	6.52	6.52	6.52	6.52
			7.42	7.45	7.45	7.45	7.45
				8.02	8.05	8.05	8.05
					8.37	8.37	8.37
						8.80	8.80
							9.20
0.025 N diethyl glutarate.							
2.37	3.50	3.55	3.55	3.55	3.55	3.55	
	3.90	4.00	4.00	4.00	4.00	4.00	
		4.85	4.85	4.90	4.90	4.90	
			5.35	5.35	5.40	5.40	
				5.62	5.62	5.62	
					5.75	5.75	
						5.85	
						5.87	

lar results were obtained with the 0.025 N solution of the ester and in other experiments not presented in the tables. We are not able to offer any adequate explanation for the difference in the course of the hydrolysis, with solutions neutralized at frequent intervals and those titrated only once. It seems probable that inactivation of the lipase may have occurred, although in experiments (3)

## Lipase. II

similarly conducted with simple esters, e.g. ethyl acetate, ethyl propionate, ethyl butyrate, etc., no such change in lipolytic activity has been noted.

*Diethyl Adipate (Table IV).*—The results obtained with diethyl adipate are comparable to those obtained with diethyl glutarate.

TABLE IV.

*Hydrolysis of 0.025 and 0.0125 N Solutions of Diethyl Adipate by Lipase.*

25 cc. portions of the ester solution were used. For complete saponification of this amount of these solutions 6.35 and 3.2 cc., respectively, of 0.09872 N NaOH were required.

## 0.025 N diethyl adipate.

Time...30 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	7 hrs.	9 hrs.	11 hrs.
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## Standard NaOH required for neutralization of acidity.

| cc.  |
|------|------|------|------|------|------|------|------|------|
| 2.70 | 3.97 | 4.12 | 4.12 | 4.12 | 4.12 | 4.12 | 4.12 | 4.12 |
|      | 3.95 | 4.45 | 4.45 | 4.45 | 4.45 | 4.45 | 4.45 | 4.45 |
|      |      | 5.55 | 5.55 | 5.55 | 5.55 | 5.55 | 5.55 | 5.55 |
|      |      |      | 5.70 | 5.70 | 5.70 | 5.70 | 5.70 | 5.70 |
|      |      |      |      | 6.10 | 6.10 | 6.10 | 6.10 | 6.10 |
|      |      |      |      |      | 6.10 | 6.10 | 6.10 | 6.10 |
|      |      |      |      |      |      | 6.30 | 6.30 | 6.30 |
|      |      |      |      |      |      |      | 6.15 | 6.15 |
|      |      |      |      |      |      |      |      | 6.15 |

## 0.0125 N diethyl adipate.

Time...30 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	8 hrs.	9 hrs.
----------------	-------	--------	--------	--------	--------	--------	--------	--------

## Standard NaOH required for neutralization of acidity.

| cc.  |
|------|------|------|------|------|------|------|------|------|
| 0.45 | 1.42 | 2.10 | 2.10 | 2.15 | 2.15 | 2.15 | 2.15 | 2.15 |
|      | 0.67 | 2.30 | 2.32 | 2.32 | 2.32 | 2.32 | 2.32 | 2.32 |
|      | .    | 1.05 | 2.45 | 2.45 | 2.45 | 2.45 | 2.45 | 2.45 |
|      |      |      | 1.85 | 2.25 | 2.25 | 2.25 | 2.25 | 2.25 |
|      |      |      |      | 2.10 | 2.55 | 2.55 | 2.55 | 2.55 |
|      |      |      |      |      | 2.25 | 2.35 | 2.35 | 2.35 |
|      |      |      |      |      |      | 3.07 | 3.07 | 3.07 |
|      |      |      |      |      |      |      | 3.35 | 3.35 |

The insolubility of the adipate necessitated the use of lower concentrations of the ester (0.025 and 0.0125 N) in order to obtain complete solution. With these dilute solutions, nearly complete

hydrolysis of the ester was obtained in 4 to 9 hours. As with diethyl glutarate, neutralization of the acidity developed checked the further hydrolysis if the acidity was neutralized after a hydrolysis of approximately 50 per cent had occurred. Experiments, in which the isolation of the products of hydrolysis of diethyl glutarate was attempted were only partially successful. We have, however, been able to demonstrate the formation of adipic acid at various stages in the hydrolysis by lipase. 4 cc. of diethyl adipate were mixed with 100 cc. of water and 5 cc. of the glycerol extract of liver added. Toluene was added and the flask allowed to stand at room temperature for 18 days. At the end of this time, 152.5 cc. of standard alkali were used to titrate the acidity developed. Inasmuch as 400.1 cc. were required for complete saponification of 4 cc. of diethyl adipate, this indicated a hydrolysis of slightly less than 40 per cent of the ester. The solution was boiled to kill the enzyme, evaporated to dryness, extracted with ether to remove any unchanged ester, acidified, and again extracted with ether. A considerable quantity of white crystalline material was obtained on the evaporation of the ether. After recrystallization the crystals melted at 146-147° (uncorrected). In a similar experiment, the acidity developed was neutralized daily and the reaction stopped when a total acidity of 205 cc. had been developed; that is, when a hydrolysis of 50 per cent had been obtained. From this, crystals melting at 147° were obtained by the method outlined. The neutral equivalent of the crystals was found to be 76. Adipic acid melts at 153° (corrected) and has a neutral equivalent of 73. These results indicated that adipic acid was formed in the hydrolysis by liver lipase even when the hydrolysis had proceeded only partially toward completion.

It is of interest to note that the rate of hydrolysis increased with the increase in molecular weight of the acid in the case of the diethyl esters investigated. Thus, under comparable conditions the hydrolyses of 0.05 N solutions of the diethyl esters of malonic, succinic, and glutaric acids, during the initial 30 minute period corresponded to 2.00, 2.67, and 3.30 cc. of standard alkali, respectively. With 0.0125 N solutions of diethyl glutarate and adipate, the similar hydrolyses were equivalent to 2.37 and 2.70 cc. of alkali. These results with dicarboxylic esters are in harmony with those of Kastle and Loevenhart (4) and others (5, 6) who observed

that the rate of hydrolysis of ethyl acetate, ethyl propionate, and ethyl butyrate by the lipases of liver and pancreas increased with the molecular weight.

Howard (7) has recently studied the lipolytic action of pancreatic extracts on dibenzyl succinate. He concluded that dibenzyl succinate also was split only to the monobenzyl ester. From a quantitative standpoint, it is difficult to accept the results of these experiments inasmuch as the ester was not in solution and the conditions were not such as to favor optimum activity of the enzyme. Horst (8) in this laboratory has studied the hydrolysis of dibenzyl succinate in the organism of the rabbit. He observed that the amount of hippuric acid present in the urine after the feeding of dibenzyl succinate indicated a complete cleavage of the ester and oxidation of the liberated benzyl alcohol to benzoic acid with subsequent conjugation and elimination as hippuric acid. These results are at variance with the *in vitro* studies of Howard. It is possible that the cleavage of diethyl succinate and malonate may be complete in the animal body and that the results reported in this and our former paper (1) have little significance for the living organism. Further experiments along these lines are in progress.

#### SUMMARY.

1. In confirmation of earlier work (1), the hydrolysis of the diethyl esters of succinic and malonic acids by lipase of hog liver was observed to proceed rapidly to an equilibrium which corresponded to the removal of one ethyl group from the diethyl ester.

2. In the hydrolysis of the diethyl esters of adipic and glutaric acids under like conditions, equilibrium was reached when the cleavage which corresponded to the removal of both the ethyl groups was nearly complete. The hydrolysis of these esters followed a course similar to that observed with the simple esters of the monocarboxylic acids.

3. Adipic acid was isolated from the products of hydrolysis of diethyl adipate by liver lipase in experiments in which the hydrolysis was less than 50 per cent complete.

4. Under comparable experimental conditions the rate of hydrolysis of the diethyl esters of malonic, succinic, glutaric, and adipic acids increased with the increase in the molecular weight of the acids.

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# VARIATIONS IN THE DISTRIBUTION OF THE NON-PROTEIN NITROGENOUS CONSTITUENTS OF WHOLE BLOOD AND PLASMA DURING ACUTE RETENTION AND ELIMINATION.

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Several writers, basing their statements on definite analytical data, have recently emphasized the fact that plasma is to be preferred to whole blood for analysis in investigating metabolic conditions, since it represents that portion of the circulating medium most directly concerned in the processes of assimilation and excretion. There are, however, remarkably few published reports dealing with the normal distribution of the constituents of the blood between the corpuscles and plasma, and this is especially true of the non-protein constituents commonly determined in the course of clinical studies on renal function.

Wu (1) has reported analyses for the usual non-protein nitrogenous constituents, as well as for sugar and sodium chloride, on the corpuscles and plasma of twenty normal human bloods. His investigations show that the total non-protein nitrogen, total creatinine, preformed creatinine, amino-acids, and sugar are normally in greater concentration in the corpuscles, while urea, uric acid, and sodium chloride are higher in the plasma. Although he gives no figures for whole blood, it is possible to calculate whole blood values from the recorded hematocrit readings and the data on plasma and corpuscles. He deprecates the common use of whole blood in the study of metabolic problems, and warmly advocates the employment of plasma for this purpose.

More recently Folin and Berglund (2) recorded the results of total non-protein nitrogen, urea nitrogen, and amino-acid nitrogen determinations on the whole blood, plasma, and corpuscles of twelve healthy young men and, at about the same time, Folin reported figures for amino-acid nitrogen, urea nitrogen, creatinine, uric acid, and total non-protein nitrogen in the whole blood, plasma, and corpuscles of seven patients with definite nitrogen retention. As a result of this experience he states that "if the different

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water content of plasma and corpuscles be disregarded, then only the amino-acids, creatine and the undetermined nitrogen are more abundant in the corpuscles than in the plasma; while the nitrogenous waste products—urea, creatinine and uric acid—are more abundant in the plasma."

In the course of some extensive work on the content of the blood in the non-protein nitrogenous substances during normal pregnancy and eclampsia, a number of analyses were made simultaneously upon whole blood and plasma, in an attempt to explain the disagreements between our results, obtained on oxalated plasma, and those of certain other investigators, who had analyzed the whole blood. In all, thirty-seven such parallel determinations were made, in sixteen of which the hematocrit values were obtained, so that it is possible in these cases to compute with considerable accuracy the content of the corpuscles in non-protein nitrogenous substances. While these figures show that there are no startling variations in the non-protein nitrogen of the blood during normal pregnancy, they are presented, with special reference to the types of cases involved, not only to emphasize the essential differences between analyses on the whole blood and plasma, but also to suggest that, in the extremely rapid changes in concentration occurring in the toxemias of pregnancy, as well as in the normal puerperium, these substances may pass from cells to plasma, or in the reverse direction, slowly enough so that the usual relationships are disturbed. The figures presented are interpreted as showing that substances, such as the non-protein nitrogenous constituents of the blood, which have accumulated in the plasma during periods of retention, may be temporarily deposited in the corpuscles, from which they are given up gradually as the plasma content of these substances is reduced by improved elimination.

### EXPERIMENTAL.

Blood was collected from an arm vein directly into a flask containing either dry powdered potassium oxalate or 1 to 2 cc. of a 5 per cent solution of this salt. Analyses were made immediately, using the micro methods of Folin and Wu for both the whole blood and plasma, without essential modification. In a short series of preliminary experiments it was ascertained that the amounts of  $\frac{2}{3}$  N sulfuric acid and 10 per cent sodium tungstate recommended

for the protein precipitation in whole blood could be used for plasma,<sup>1</sup> providing the reaction of the filtrate were made neutral to methyl orange before attempting the urea determination. Hematocrit readings were made after centrifuging the oxalated blood at high speed (3,000 to 3,500 R.P.M.) until the precipitated cells maintained a constant volume.

The results of the twenty-one determinations in which no hematocrit readings were obtained are presented in Table I. Although they represent specimens of blood from only six individuals, as many as seven samples having been obtained at daily intervals from one patient, they present, nevertheless, quite a wide range of values, because of the pathological conditions which obtained in certain cases. From this table, the following facts will be noted.

The total non-protein nitrogen is higher in the whole blood in every case but one, the range of variation being from 1 to 10 mg. per 100 cc.

The urea nitrogen is generally slightly higher in the plasma, although occasionally the reverse is true.

The relation of the urea nitrogen to the total non-protein nitrogen is in accord with the absolute values for these fractions, and in three instances only (Case 2, Specimen 1; Case 6, Specimens 5 and 6) are the urea percentages in the whole blood higher than in the plasma. From the conditions which prevailed when these unusual values were obtained, we are inclined to regard them as representing an actual reversal of the normal relationship between plasma and corpuscles, rather than as analytical errors.

Uric acid is usually more concentrated in the plasma, but during periods of the rapid disappearance of uric acid retention in the blood, these values may be reversed for a short period, just as the usual difference in favor of the plasma may be exaggerated when increased retention is in progress.

As has been previously recorded, the preformed creatinine is generally somewhat higher in the whole blood; while creatine (total minus preformed creatinine) is, of course, considerably more concentrated in the corpuscles.

<sup>1</sup> Wu (1) practically agrees with this, although he recommends the use of one-half of the whole blood quantities of the two precipitants for analyses of the plasma.

TABLE I.  
*Whole Blood and Plasma Analyses.*

Case No.	Hospital No.	Total non-protein N.	Urea N.	Ureic acid.	Creatinin.	Creatine.	Remarks.	
		Whole blood. Plasma. mg. per 100 cc.						
1	10,757	39	29	19	47	65	2.6	3.5
2	10,844	24	23	13	12	55	2.9	3.1
3	10,797	25	21	11	12	42	2.1	2.3
4	10,787	34	26	18	21	54	3.3	4.5
	.	36	28	20	22	55	2.9	3.1
	.	37	30	17	25	47	2.6	2.8
4	76	83	60	60	72	79	6.6	6.5
		99	96	71	77	72	8.8	10.4
		45	38	23	27	52	70	6.3
5	10,769	46	41	21	24	47	58	9.4
		41	34	25	23	62	68	7.5
		42	34	26	25	62	75	4.9
		44	34	22	22	51	64	4.3

10,792	23	17	11	12	48	71	1.5	1.8	1.4	1.1	4.2	1.7
	24	19	13	14	53	73	1.4	1.2	1.4	1.2	5.3	2.0
	27	19	10	11	39	55	0.9	0.8	1.5	1.2	3.8	3.9
	21	19	11	12	53	62	0.6	0.5	1.2	1.0	4.4	1.5
	23	20	10	8	41	37	0.7	0.8	1.4	1.4	4.0	1.2
	24	19	11	9	47	46	0.8	0.9	1.5	1.5	3.7	0.9
	23	17	10	8	43	48	0.8	0.7	1.3	1.2	4.9	0.9

TABLE II.  
*Whole Blood and Plasma Analyses with Calculated Corpuscle Values.*

Table II presents the total non-protein nitrogen, urea nitrogen, uric acid content, and the urea N: total non-protein N ratio of sixteen specimens of whole blood and plasma, together with the respective values for corpuscles, calculated from the hematocrit readings.

Here the differences between whole blood and plasma agree in general with those noted from Table I, and the few variations are in line with those already noted. The total non-protein nitrogen of the corpuscles is very high as compared with that of the plasma. The urea values are usually higher in the plasma than in the corpuscles, although in the cases where the whole blood figures are higher than those of the plasma, this difference is exaggerated in the corpuscles. The uric acid content of the plasma is, with two exceptions, higher than that of the corpuscles.

#### DISCUSSION.

It has, until very recently, been tacitly assumed that study of the whole blood would give the same information generally as would examination of either of its component parts, since most substances can pass readily from one to the other by simple diffusion. The possibility of an appreciable latent period has, however, been generally overlooked. One recognized exception to the usual approximately even balance is creatine, which is admitted to be contained almost exclusively in the corpuscles, and to be present in small quantities in the plasma only when there is a creatinuria. The work of Wu, and of Folin and Berglund, to which reference has been made, demonstrates that conditions are not so simple, and that it is quite normal for the non-protein constituents to be at different concentrations in the corpuscles and in their surrounding plasma. The evidence which these authors submit would, however, indicate that these relationships are quite fixed, whereas the decided variations from the normal which we have observed during periods of retention or rapid elimination, surely demand attention.

We have evidence,<sup>2</sup> which will be reported elsewhere, to show that, while there is usually only a slight degree of retention of the non-protein nitrogenous material of the blood (whole blood or

<sup>2</sup> Reported briefly in the Transactions of the Association for Research in Nervous and Mental Disease in December, 1922. To be reported in full elsewhere.

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plasma) at the height of the so called "late toxemias of pregnancy," the period of improvement is marked by a rapid piling-up of these materials, followed immediately by an abrupt drop to normal. Under such circumstances, conditions in the plasma must be changing with relatively great rapidity and it would not be surprising if diffusion into or out of the corpuscles failed to keep pace with the retention or elimination. From the data presented in the tables it appears that there is a proportionate increase in the non-protein nitrogenous material in the plasma during active retention, while there is proportionate increase in these same substances in the blood cells during the rapid reduction of such a retention. There follows a more detailed discussion of the conditions under which such variations from the normal were noted.

*Total Non-Protein Nitrogen.*—In only one specimen (Case 4, Specimen 2) among the thirty-seven reported was the total non-protein nitrogen higher in the plasma than in the whole blood. This blood was taken from a patient with outspoken chronic nephritis, who developed such severe symptoms when 5 months pregnant that the uterus was emptied by abdominal hysterotomy and tubal sterilization effected. The first analyses, made upon admission, showed only a slight grade of retention, with the urea nitrogen disproportionately high. 3 days later, 25 hours after the operation, all the non-protein nitrogenous constituents had increased in amount from twice to nearly four times and the whole blood and plasma values were practically identical, with the exception of the total non-protein nitrogen, which was higher in the plasma. In view of the other evidence to be adduced later, the explanation probably lies in the fact that some portion of the undetermined nitrogen fraction was released into the plasma (presumably from the tissues) more rapidly than it could be excreted, and more rapidly than it could come into equilibrium with the blood cells. The next specimen, taken 3 days later, contained large amounts of all non-protein nitrogenous constituents, but the total non-protein nitrogen of the plasma was restored to its normal relationship with that of the corpuscles, being higher in the whole blood than in the plasma. It may be assumed that the high point of the retention had been passed by the time this sample was obtained.

*Urea Nitrogen.*—In nine of the thirty-seven specimens, the urea nitrogen was slightly higher in the whole blood than in the plasma, so that the corpuscles must have actually contained considerably more urea than the plasma. It is apparent from the data appended under "Remarks" that all these specimens were obtained during periods when a profuse diuresis was to be expected either normally (Case 6), or during convalescence from eclampsia (Cases 5 and 14). Fig. 1 is a graphic representation of the changes observed in Case 14, and shows that on the last 2 days of the experiment, when the values for non-protein nitrogenous constituents were rapidly returning to normal, the urea was in higher concentration in the corpuscles than in the plasma. Although this is directly opposed to the statement of Folin (3) that, "Both normally and in nitrogen retentions the urea is more abundant in the plasma than in the corpuscles," the same condition was observed in several other patients under similar circumstances. Moreover, in a few instances during periods of rapid retention, the opposite condition was observed—the usual difference between whole blood and plasma urea was exaggerated with the result that the latter contained 4 to 8 mg. per 100 cc. more urea nitrogen than did the whole blood. It is obvious that the differences between corpuscles and plasma were even more significant. Case 10 illustrates this magnified difference.

*Urea Nitrogen : Total Non-Protein Nitrogen Ratio.*—The urea nitrogen forms a larger percentage of the total non-protein nitrogen in the plasma than in the whole blood, because of the smaller absolute amounts of undetermined nitrogen. We have not encountered the very low percentages reported for normal pregnancy by Folin (4), a failure which has been reported by others. In Case 2, Specimen 1, and in Case 6, Specimens 5 and 6, the higher urea nitrogen:total non-protein nitrogen ratios in the whole blood are associated with disturbances of the total non-protein nitrogen similar to those previously noted. The curves of the urea percentages in Fig. 1 are suggestive, but their interpretation is not clear; although it is apparent that the non-protein nitrogenous materials, other than urea, which make up such a large part of the total non-protein nitrogen of the corpuscles during the acute toxemia, are eliminated more rapidly than the urea, so that the percentage of the latter soon rises to normal.

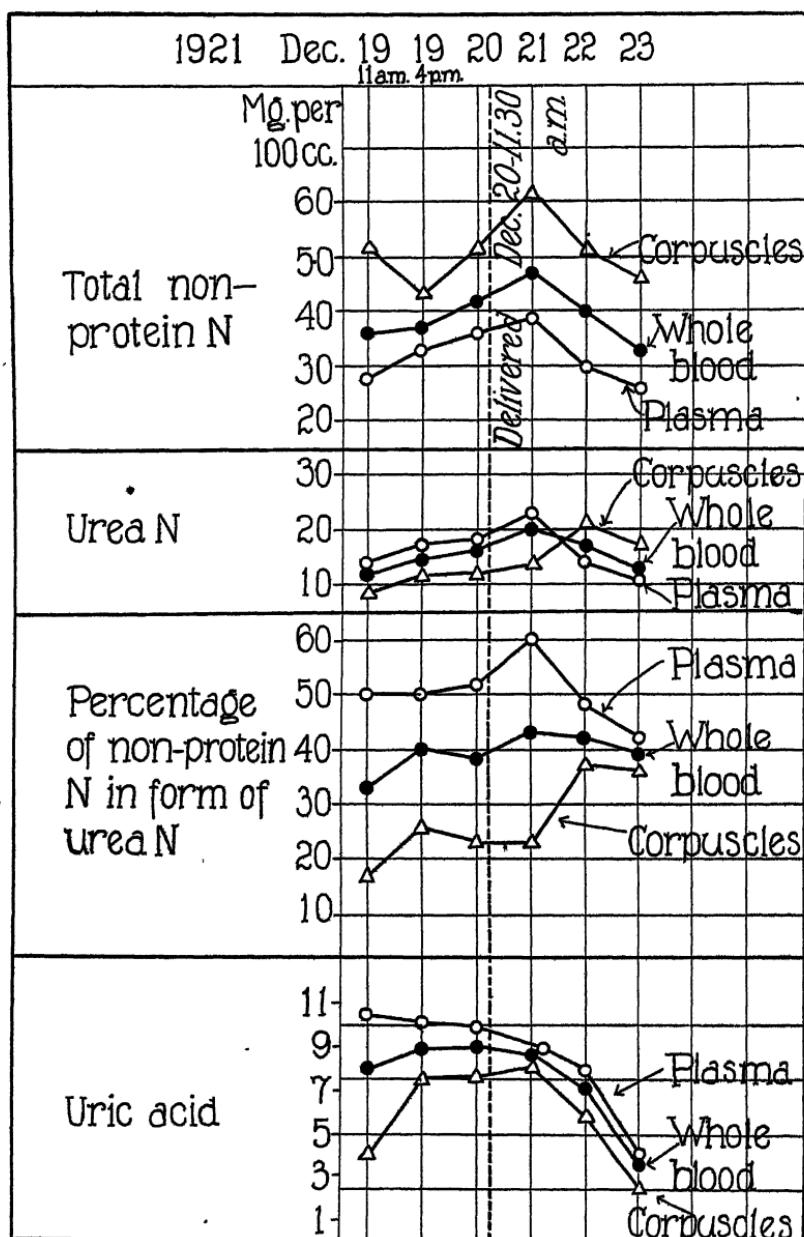


FIG. 1. Graphic chart of results in Case 14. Antepartum eclampsia treated with morphine.

*Uric Acid.*—Uric acid is known to be in higher concentration normally in the plasma than in the whole blood and Folin (3) even makes the statement that, "In the case of uric acid the difference between the figures obtained from whole blood and from plasma is so large that practically the whole of the uric acid content of the blood falls on the plasma." We cannot subscribe to this opinion because we have examined several specimens in which the whole blood showed more uric acid than the plasma, indicating that the corpuscles contained proportionately more of this substance. Here, too, the actual relation between the blood cells and the plasma is apparently liable to sudden changes in periods of rapid retention or elimination. For example, in Fig. 1 it is apparent that the uric acid first accumulated in the plasma, but that it rather quickly (5 hours) diffused into the corpuscles, so that the whole blood and plasma values became nearly identical. Under normal conditions, it is agreed that the corpuscles are relatively poor in uric acid, but when, on the other hand, an acute retention is in progress, this substance first increases in the plasma and is then gradually absorbed into the corpuscles until nearly equal values obtain. Our figures also suggest that during the rapid reduction of a retention, the plasma may lose its uric acid through the kidneys so rapidly that the influx from the corpuscles cannot keep pace, and the whole blood temporarily contains more uric acid than the plasma.

*Creatinine and Creatine.*—Determinations of creatinine were done on such a small number of specimens that general statements are dangerous. The preformed creatinine values are within the usually accepted normal range, while the whole blood figures, if anything, are slightly higher than those of the plasma. The status of whole blood creatine (total minus preformed creatinine) is still so unsettled that statements concerning it have at best a doubtful value. The plasma determination is on a better footing, however, so that one may compare the values obtained, if only it is not insisted too strongly that the figures are for *creatine*.

Plasma creatine was determined in Cases 3, 4, and 6 and it is noticeable that the values tend to rise after delivery and then return to normal. This increase in the creatine in the plasma occurs at the time when the urinary output of this substance is most marked (5). In one case (No. 6) the total urinary creatine

output was determined simultaneously with the blood content and the curves of the plasma and urine creatine are plotted in Fig. 2.

Wilson and Plass (6) have postulated the hypothesis that creatine is an inconstant constituent of blood plasma, which is always excreted in the urine if present in the plasma—the normal threshold being zero. The fact that, during the puerperium, the pronounced creatinuria is accompanied by an increased amount of creatine in the plasma, is quite in accord with their observations.

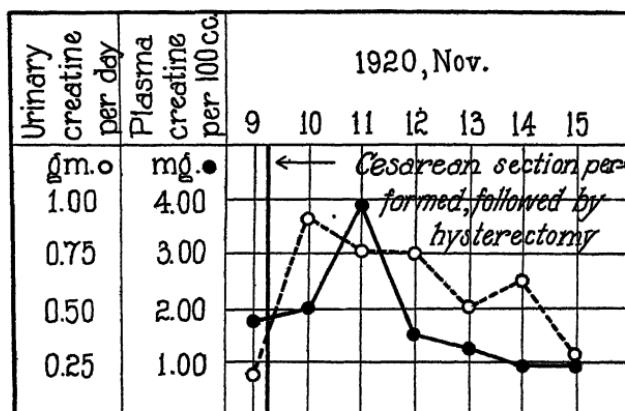


FIG. 2. Plasma and urine creatine during the puerperium.

It is suggestive that in Case 6 the whole blood creatine diminishes as the plasma creatine reaches its maximum, but, in the absence of other confirmatory observations, it is doubtful whether it can be safely assumed that the excess detected in the plasma has come from the corpuscles, although further work may show that to be the case.

#### SUMMARY.

The extremely rapid changes in concentration of the non-protein nitrogenous constituents of the blood during recovery from the so called "late toxemias of pregnancy," have been studied by simultaneous analyses of whole blood and plasma, and it has been demonstrated that these substances pass from cells to plasma, and in the reverse direction, so slowly, that at times the usual

relationships are disturbed. It is particularly emphasized that the blood corpuscles are storehouses into which material accumulated in the plasma may be temporarily deposited, and from which the excess may later be rapidly removed. Under such conditions it is obviously important to attack the study of certain metabolic problems from the standpoint of the blood cells as well as of the plasma.

#### CONCLUSIONS.

1. The total non-protein nitrogen is usually higher in the whole blood than in the plasma, but occasionally during very rapid retention the corpuscles may not absorb these substances as rapidly as they are accumulated in the plasma, so that the latter temporarily has a higher concentration.
2. Urea is ordinarily slightly higher in the plasma, although this relationship may be altered in either direction. During periods of rapid retention, the difference in favor of the plasma may become exaggerated, while during a rapid relief of this piling-up, the whole blood may for a time have more urea, because the rapid excretion from the plasma is not accompanied by an equally rapid diffusion out of the corpuscles.
3. *Uric acid* accumulates first in the plasma under conditions of retention, but later diffuses into the blood cells, until they come into practical equilibrium. During the precipitate relief of such a retention, the corpuscles may at times actually contain more uric acid than the plasma.
4. Creatine, which is normally absent from the plasma, is present in higher concentration during the period of puerperal creatinuria, so that the curves of plasma and urinary creatine are quite similar.

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## CHEMICAL CONSTITUENTS OF SALIVA AS INDICES OF GLANDULAR ACTIVITY.\*

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Study of the uric acid content of the secretory products of the digestive glands was made possible when the accurate method of Morris and Macleod<sup>1</sup> for the determination of uric acid in small quantities became available about a year and a half ago. Since that time data have been collected, as opportunity offered, and an effort made to interpret the results in relation to the part the glands play in endogenous metabolism. The difficulty of obtaining the secretions from most of the digestive glands has delayed the completion of the work planned. The accessibility of the salivary glands and ease of collecting their secretion made possible the greatest progress. Recent publications of Starr<sup>2</sup> on the hydrogen ion concentration of the saliva, Hench and Aldrich,<sup>3</sup> and Schmitz<sup>4</sup> on the salivary urea content made it seem advisable to publish representative chemical data secured by the authors in their study of the activity of the salivary glands. Data on certain of the other glands will be published later.

The present communication includes extensive data for salivary urea, ammonia, amino-acids, and creatinine in addition to those on uric acid. The former were first required for their value in interpretations in the salivary uric acid content under differing

\* A preliminary report of this work was presented before the American Society of Biological Chemists, December 28, 1922 (cf. Morris, J. L., and Jersey, V., *J. Biol. Chem.*, 1923, iv, p. xviii).

<sup>1</sup> Morris, J. L., and Macleod, A. G., *J. Biol. Chem.*, 1922, I, 55.

<sup>2</sup> Starr, H. E., *J. Biol. Chem.*, 1922, liv, 43.

<sup>3</sup> Hench, P. S., and Aldrich, M., *J. Am. Med. Assn.*, 1922, lxxix, 1409.

<sup>4</sup> Schmitz, H. W., *J. Lab. and Clin. Med.*, 1922-23, viii, 78.

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conditions. Later, they were secured for the information they contribute in the study of the glandular activity. Finally, all the data bear on the question of the value of saliva analysis as a diagnostic procedure.

Of no small importance in these problems is the method of collecting the saliva specimen. The secretion is much less subject to irregularities in chemical content when collected from the quiescent or resting glands than when obtained from the stimulated glands. In order to increase the quantity of saliva it has been customary in previous investigations for the subject to chew paraffin. Our experience indicates that this method introduces marked variations in volume and chemical content which makes the method quite unsatisfactory where the secretion is to be analyzed quantitatively. Throughout the work here presented the collection is from the resting glands and the output is measured in time intervals. This method, at least to a great extent, avoids the difficulties that result from paraffin chewing.

In Table I is a series of such collections, showing the relative uniformity of volume and chemical content of several consecutive half hour specimens contrasted with a half hour (11.00 to 11.30 a.m.) collection which made use of paraffin chewing. The figures representing the half hour output of the resting glands (first column for each constituent) are more uniform than are the figures (second column in each case) which express the same values in terms of 100 cc. of secretion. The increases due to paraffin chewing differ in amount for the various constituents, the urea plus ammonia nitrogen value paralleling the volume most nearly, the uric acid least. For this reason the figures for urea calculated for 100 cc. are least disturbed by the stimulus of chewing, the corresponding figures for uric acid most. Of the constituents so far studied urea plus ammonia nitrogen values expressed in milligrams per 100 cc. most nearly approach the accuracy of the figures based on the time factor, and may be considered sufficiently accurate for diagnostic purposes; while uric acid certainly cannot be so measured. Amino-acid nitrogen and creatinine lie between these extremes. The extent of these variations cannot be indicated with any definiteness. They differ with the vigor of the chewing. Reference to the uric acid values in Table II makes this apparent. V. J. (adult) chewing paraffin at his usual rate, had

TABLE I.

*Secretion from Salivary Glands When Resting and under Stimulus of Paraffin Chewing.*

Time.	Vol- ume of saliva.	Urea plus ammonia nitrogen.*		Amino-acid nitrogen.*		Creatinine.*		Uric acid.*	
		cc.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
7.00- 7.30	11.0	2.24	20.4	1.10	10.0	0.10	0.86	0.39	3.54
7.30- 8.00	13.0	2.58	21.8	1.09	8.4	0.10	0.78	0.53	4.00
8.00- 8.30	15.8	2.18	13.8	1.24	7.9	0.10	0.66	0.57	3.55
8.30- 9.00 Breakfast.									
9.00- 9.30	12.5	2.16	17.3	0.76	6.1	0.10	0.83	0.52	4.16
9.30-10.00	20.5	2.42	11.8	1.09	5.4	0.14	0.68	0.64	3.10
10.00-10.30	19.8	2.54	12.4	1.02	5.2	0.13	0.66	0.69	3.44
10.30-11.00	22.5	2.91	12.9	1.20	5.3	0.14	0.64	0.70	3.11
11.00-11.30†	119.0	12.52	10.5	9.86	8.8	0.31	0.26	1.14	0.96
11.30-12.00	23.0	2.69	11.7	0.93	4.0	0.13	0.54	0.62	2.65
p.m.									
12.00-12.30	21.2	2.85	13.4	1.16	5.5	0.12	0.56	0.60	2.83

\* The figures in the first column for each constituent represent quantities per half hour. The figures in the second column represent quantities calculated for 100 cc. of saliva.

† Paraffin chewed from 11.00 to 11.30 a.m.

TABLE II.

*Saliva Collection Methods Compared.*

Subject.	Age.	Paraffin chewed.		Natural secretion.
		yrs.	mg.	
V. J.	Adult.		1.44	3.5
			1.62	4.2
J. L. M.	"			3.9
			2.04	4.0
			1.48	3.6
J. S.	8			3.3
			0.96	
L. E. M.	7			2.7
W. S.	10			3.7

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1.44 and 1.62 mg. of uric acid per 100 cc. Saliva from J. S. (8 year old boy) under similar conditions had 0.92 mg. per 100 cc. In the case of J. L. M. (adult) his usual rate of chewing gave a value of 1.48 mg. per 100 cc. When the last subject chewed about half as rapidly as usual, the uric acid figure was 2.04 mg.; when he chewed about twice as fast as usual, the figure was 0.96 mg. The last figure is as low as the usual value for the 7 and 8 year old boys. Obviously, the vigor of chewing plays a very considerable part in the average figures given in the literature for salivary uric acid.<sup>5</sup> The uric acid values determined for the natural secretion of the resting glands of the same subjects (Table II) are much more uniform. Paraffin collection introduces errors into the analysis of the other salivary chemical constituents also, but they are less exaggerated than in the case of uric acid. Throughout the present study saliva was collected in half hour intervals from the quiescent glands. The following methods of collection, preparation of the specimens, and analysis were used.

*Collection of Specimen.*—Place a small funnel (1 inch diameter) behind the lips and against the teeth, and hold a graduated cylinder over the stem to catch the secretion. Keep the head bent slightly forward and refrain from chewing or swallowing. Change the cylinder each half hour during the series, thus collecting consecutive specimens (four to twenty-five in the work here presented).

*Preparation for Analysis.*—Note the volume of each half hour quantity (usually 5 to 25 cc.) and transfer to a 100 cc. volumetric flask (200 cc. in those cases where the half hour output, due to some vigorous stimulus, is more than 50 cc.). Add 2.5 cc. of 2 per cent acetic acid and shake. Now add 10 cc. of 10 per cent sodium sulfate and sufficient water to make the total volume about 75 cc. Finally, add 10 cc. of alumina cream and make up to volume. Shake from time to time during 10 minutes and filter.

<sup>5</sup> Average figures of 2.1 mg. for men and 1.1 mg. for women were reported by Lowenstein and Gies (Lowenstein, G. A., and Gies, W. J., *Proc. Soc. Exp. Biol. and Med.*, 1918-19, xvi, 53). Similar values (0.6 to 2.9 mg. for men and 0.7 to 2.3 for women) were reported by Lewis and Updegraff (Lewis, H. B., and Updegraff, H., *Proc. Soc. Exp. Biol. and Med.*, 1922-23, xx, 168).

Double the quantites when a 200 cc. flask is required. Filtrates so prepared have been perfectly clear. Any traces of protein remaining did not interfere with any of the determinations made.

*Chemical Methods.*—Urea plus ammonia nitrogen, ammonia nitrogen, amino-acid nitrogen, and creatinine were determined by the Folin-Wu<sup>6</sup> blood methods, their application to the saliva filtrates proving entirely satisfactory. Uric acid was determined by the Morris and Macleod blood method. (Preliminary work

TABLE III.  
8 Hour Series of Half Hour Specimens.

Time.	Volume of saliva.	Urea plus ammonia nitrogen.*		Ammonia nitrogen.*		Amino-acid nitrogen.*		Creatinine.*		Uric acid.*	
a.m.	cc.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
8.00- 8.30	14.0	2.41	17.2	1.71	12.2	1.58	11.3	0.15	1.09	0.47	3.36
8.30- 9.00	18.2	2.94	16.1	2.03	11.2	1.71	9.4	0.18	0.97	0.62	3.40
9.00- 9.30	20.4	2.83	13.9	1.38	6.8	1.77	8.7	0.15	0.72	0.57	2.79
9.30-10.00	19.5	2.85	14.6	1.76	9.0	1.63	8.4	0.14	0.70	0.60	3.04
10.00-10.30	19.7	3.03	15.4	2.11	10.7	1.71	8.7	0.14	0.73	0.61	3.09
10.30-11.00	21.5	3.03	14.1	2.09	9.7	1.65	7.7	0.16	0.73	0.56	2.60
11.00-11.30	24.4	3.57	14.6	3.00	12.3	1.95	8.0	0.17	0.68	0.66	2.70
11.30-12.00	19.8	2.85	14.4	2.39	12.1	1.51	7.6	0.16	0.82	0.60	3.03
p.m.											
12.30-1.00	20.3	3.03	14.9	1.98	9.8	1.49	7.3	0.14	0.67	0.53	2.61
1.00-1.30	19.5	3.06	15.7	2.01	10.3	1.44	7.4	0.15	0.75	0.52	2.67
1.30-2.00	20.2	2.88	14.3	2.04	10.1	1.48	7.3	0.15	0.74	0.51	2.52
2.00-2.30	19.0	2.40	12.6	1.92	10.1	1.41	7.4	0.14	0.75	0.51	2.68
2.30-3.00	22.5	3.80	16.9	2.66	11.8	1.79	8.0	0.13	0.58	0.71	3.15
3.00-3.30	20.3	3.33	16.4	2.44	12.0	1.75	8.6	0.15	0.75	0.68	3.36
3.30-4.00	27.2	3.71	18.6	2.61	9.6	2.03	7.5	0.17	0.61	0.82	3.01

\* The figures in the first column for each constituent represent quantities per half hour. The figures in the second column represent quantities calculated for 100 cc. of saliva.

on many saliva specimens gave results by the Folin-Wu and Morris-Macleod methods which agreed, warranting the conclusion that uric acid alone was represented.)

Attempts to use the Folin-Wu<sup>7</sup> method for the determination of glucose resulted uniformly in the absence of more color than a

<sup>6</sup> Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

<sup>7</sup> Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, xli, 367.

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blank gives. No reducing sugar is present in saliva either from the resting glands or as a result of any of the stimuli here used.

Table III presents the values for volume and chemical constituents obtained in an 8 hour series of half hour specimens.

TABLE IV.  
*Uric Acid in Saliva during Day on Non-Purine Diet.*

Time.	Volume of saliva.	Uric acid per half hr.	Uric acid per 100 cc.
<i>a.m.</i>			
7.00- 7.30	2.8	0.15	5.36
7.30- 8.00	5.7	0.42	7.37
8.00- 8.30	4.5	0.21	4.67
8.30- 9.00 Breakfast.			
9.00- 9.30	3.5	0.20	5.65
9.30-10.00	6.8	0.39	5.74
10.00-10.30	5.4	0.29	5.28
10.30-11.00	6.7	0.37	5.59
11.00-11.30	5.2	0.26	5.00
11.30-12.00	7.6	0.35	4.71
<i>p.m.</i>			
12.00-12.30	12.1	0.44	3.66
12.30- 1.00	11.2	0.40	3.60
1.00- 1.30 Lunch.			
1.30- 2.00	10.0	0.30	3.00
2.00- 2.30	11.3	0.31	2.81
2.30- 3.00	7.4	0.22	2.97
3.00- 3.30	8.2	0.26	3.22
3.30- 4.00	14.0	0.46	3.34
4.00- 4.30	11.8	0.37	3.16
4.30- 5.00	14.2	0.42	2.98
5.00- 5.30	14.2	0.44	3.12
5.30- 6.00 Dinner.			
6.00- 6.30	10.6	0.38	3.61
6.30- 7.00	17.6	0.55	3.18
7.00- 7.30	18.2	0.49	2.70
7.30- 8.00	15.5	0.40	2.58
8.00- 8.30	11.3	0.37	3.32
8.30- 9.00	16.0	0.41	2.64

Ammonia nitrogen values are noticeably less regular than the other substances. This irregularity is characteristic of the ammonia in all series and indicates that it is not a direct product of the glands, but is formed after the secretion leaves the glands,

probably through hydrolysis of urea as suggested by Hench and Aldrich. The other four substances follow the volume of the saliva (from the resting gland) with their usual degree of uniformity. A general rise is noted in all values as the series continues. All the following series show this same tendency, with the fact emphasized, of course, in the longer series. It is especially apparent in Table IV which presents the half hour volumes and uric acid contents of 14 consecutive hours of quiescent secretion

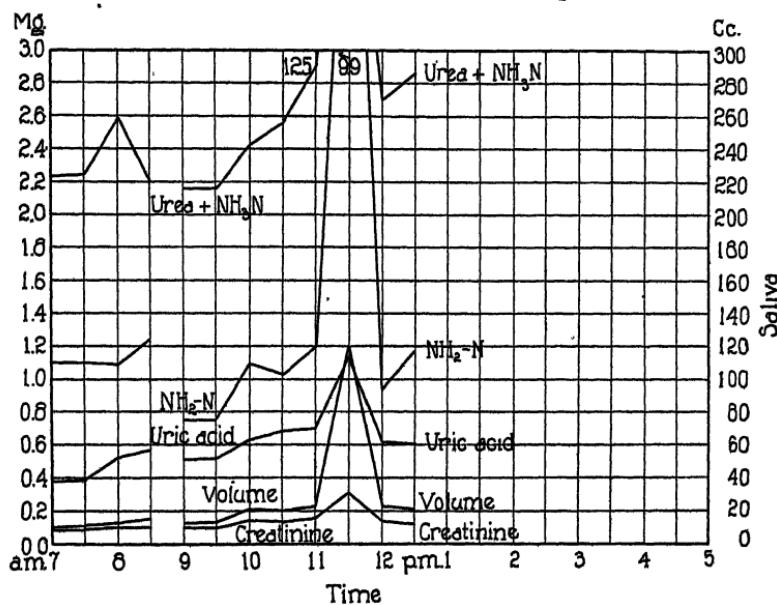


CHART 1.

Paraffin chewed 11.00-11.30 a.m.

Resulting increases:

	per cent
Volume .....	429
NH <sub>4</sub> -N .....	722
Urea + NH <sub>3</sub> N .....	374
Creatinine .....	118
Uric acid .....	63

(with three breaks of half an hour each for the meals of a non-purine diet). Rising above the local irregularities and the more general constancy of the volume and uric acid figures, there is very definite evidence that the activity of the quiescent glands increased as the day advanced. Reference to graphic records of the other

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experimental series presented in this paper proves the general nature of the phenomenon. Apparently, the increase is an index of the physiological activity involved.

On the basis of the demonstrated character of the secretion of the salivary glands in the normal resting state, the effects of various stimuli were studied. Paraffin chewing is a mechanical

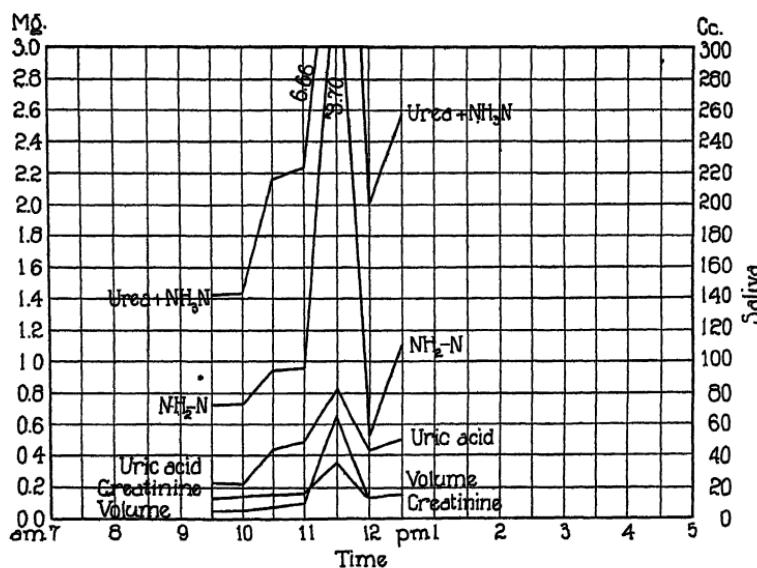


CHART 2.  
Acetic acid 11.00-11.30 a.m.  
Resulting increases:

	per cent
Volume .....	560
NH <sub>3</sub> - N.....	302
Urea + NH <sub>3</sub> N .....	200
Creatinine .....	124
Uric acid.....	71

stimulus. Its effect is expressed graphically in Chart 1. A volume increase of 429 per cent was accompanied by increases in the constituents analyzed as follows: amino-acid nitrogen, 722 per cent; urea plus ammonia nitrogen, 374 per cent; creatinine, 118 per cent; and uric acid, 63 per cent. In the series represented in Chart 2, the stimulation was due to the presence of acetic acid in the mouth (1 cc. of 2 per cent acid dropped into the mouth every 6 minutes). The volume increased 560 per cent; amino-acid nitrogen, 302 per cent; urea plus ammonia nitrogen, 200 per cent; creatinine, 124 per cent; and uric acid, 71 per

cent. Though the general effect resembles that of paraffin chewing, certain points of difference were observed. The volume increase was much greater than before, amino-acid nitrogen and urea plus ammonia nitrogen increases were about half the former

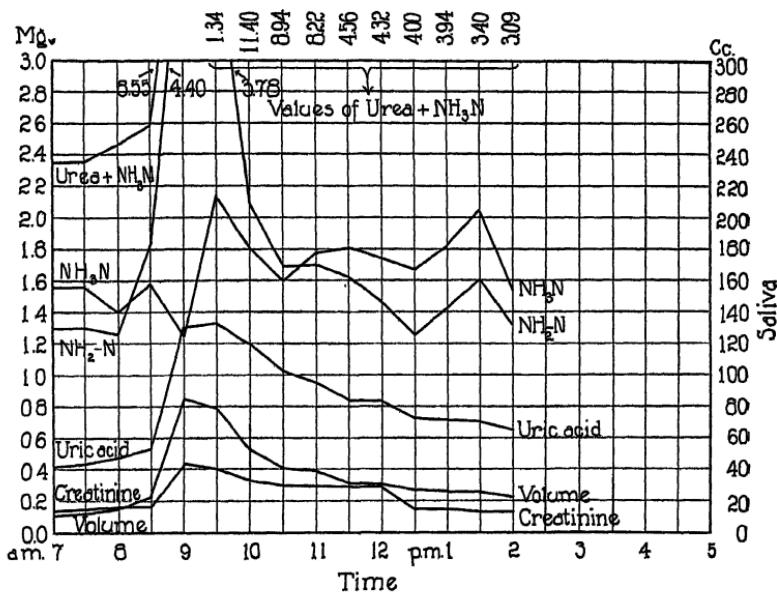


CHART 3.

Pilocarpin 8.00 a.m.;  $\frac{1}{2}$  gr.

Resulting increases:

	per cent
Volume .....	502
NH <sub>4</sub> -N .....	249
Urea + NH <sub>3</sub> N .....	445
Uric acid .....	183
Creatinine .....	171

amount while creatinine and uric acid were increased only slightly more than with paraffin chewing. Evidently, the acid brings about the pouring out of a greater volume of secretion, but the constituents are washed out with it to a less extent than in paraffin chewing. Also, it is the group which ordinarily follows the volume more closely (*i.e.* urea plus ammonia and amino-acids), which lagged markedly in this case. Taken together with the fact that uric acid and creatinine increased more than in the paraffin series, this might suggest that the two stimuli act upon different parts of the secretory mechanism. Possibly acetic acid acts more

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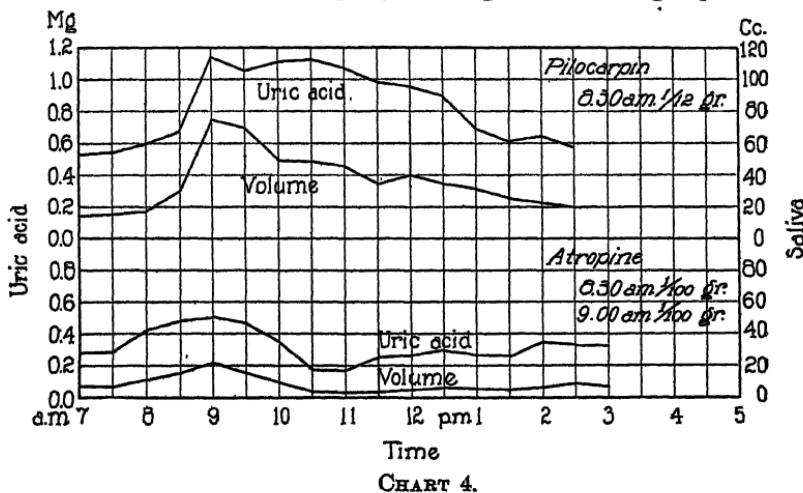
locally upon the glands while chewing exerts a general accelerating effect upon the entire process of filtration from the blood.

Further evidence in favor of an interpretation of this kind appears in the data of a pilocarpin series, graphically represented in Chart 3. After two normal half hour specimens had been collected,  $\frac{1}{16}$  grain of pilocarpin was taken by mouth. The volume rose to its maximum (84.4 cc.) an hour later, with an increase of 502 per cent. Urea plus ammonia nitrogen reached its maximum with an increase of 445 per cent. Amino-acid nitrogen increased 249 per cent. Creatinine increased 171 per cent, somewhat more than in the other series. Uric acid increased 183 per cent. The direct action of pilocarpin upon the glands differs not only from the mechanical stimulation of chewing paraffin, but also from the indirect action of an acid solution in the mouth. Amino-acids are influenced less by pilocarpin than by either of the other two stimuli, while uric acid and creatinine respond to a greater extent. Urea in this as in the paraffin series increases with the volume more closely than under the stimulation of acetic acid.

The pilocarpin-stimulating effect, though diminishing, continued for some hours. Reference to the curves (Chart 3) shows that amino-acids returned to the normal level more rapidly even than the volume. This fact and a relatively smaller increase with pilocarpin are in marked contrast with the response to paraffin chewing and acetic acid stimulation. The other constituents decreased less rapidly than the volume. This delayed return or sustained elevation is most evident in the case of uric acid. Another series of uric acid values under stimulation of pilocarpin was obtained (first set of curves in Chart 4). The same behavior of volume and uric acid values is observed with the uric acid elevation even more prolonged as the volume decreased. Without offering an explanation for this, the facts seem to warrant the statement that the larger quantity of uric acid results from some stimulating effect of the pilocarpin upon the glandular mechanism which differs from its action upon the volume of liquid produced.

Ingestion of atropine affects the volume and uric acid to a very similar degree. In the experiment, represented in the second set of curves of Chart 4,  $\frac{1}{16}$  grain of atropine was taken by mouth. The resultant paralysis of the glands decreased the volume from 20 to 2.5 cc. (88 per cent fall) and the uric acid from 0.5 to 0.17 mg. (66 per cent decrease). The return to normal values of uric acid paralleled the gradual increase of volume.

Extremes in diet evidently bring no definite changes in volume or uric acid content. Data were collected from two subjects when fasting and when eating non-purine and purine diets. The results are shown in Charts 5 and 6. The usual increase in volume and uric acid as the day advanced, took place in all six series. When non-purine food was eaten, the increases of the day were slightly less than on the fasting days. When purine food was eaten, the increases were slightly greater. Certainly there is little evidence that presence of food in the stomach has any effect in increasing the secretory activity of the salivary glands. It is also obvious that the conditions of increased uric acid elimination through the kidneys (after ingestion of large quantities



of purine-rich food) are not duplicated to any noticeable degree in the filtration through the salivary glands.

Evidently, the variations observed in saliva secretion as a result of stimuli of different kinds bear definite relations to the nature of the activating forces. Final interpretations are not yet warranted, but the selective effects of the stimuli indicate that several factors are involved in the elaboration of the secretion and that these are stressed in different proportions under various stimuli. The factors concerned may be roughly grouped as relating more directly to filtration or metabolism of the glandular tissue. Work, planned to give further evidence leading to interpretation of the problems connected with the elaboration of saliva and the conditions controlling the chemical content, is being carried out by this laboratory on normal and pathological

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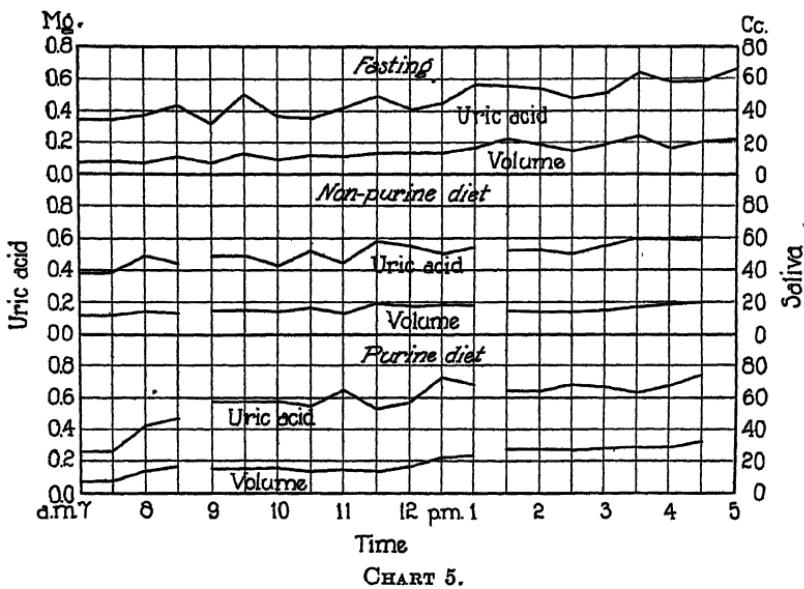


CHART 5.

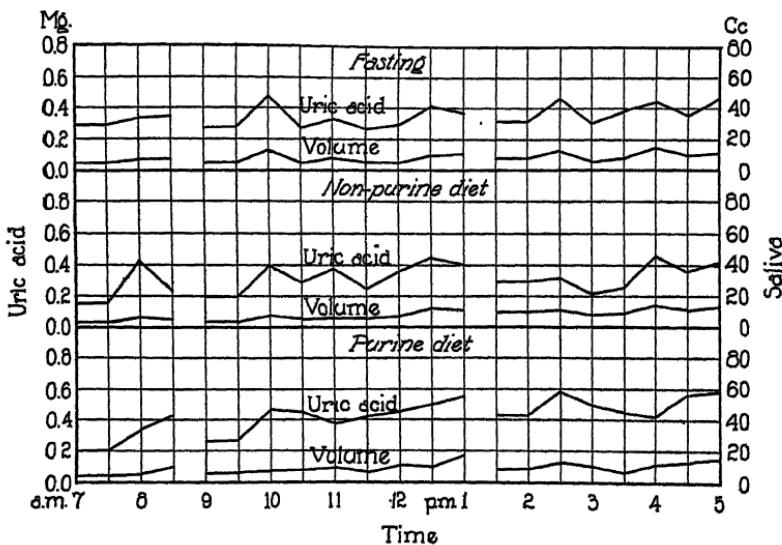


CHART 6. Paraffin chewed and saliva swallowed at 8.30 to 9.00 a.m. and 12.30 to 1 p.m.

subjects. The data in the present communication seem to justify the working hypothesis that uric acid, more than any other constituent, represents the actual cellular activity and might well serve as an index of the gland metabolism.

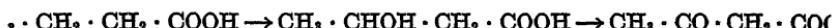
## EXPERIMENTS ON THE CATABOLISM OF CAPROIC ACID AND ITS DERIVATIVES.

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(Received for publication, March 9, 1923.)

The present paper contains the results of experiments aiming at obtaining evidence of the modes of catabolism followed by normal fatty acids when undergoing so called  $\beta$ -oxidation in the animal body. Knoop's theory of  $\beta$ -oxidation simply postulates the removal of successive pairs of carbon atoms without indicating the mechanism of the process. The common excretion of  $\beta$ -hydroxybutyric acid with acetoacetic acid in cases of faulty fatty acid catabolism, especially after administration of butyric acid, at first led to the natural assumption that the saturated fatty acid was successively oxidized to the  $\beta$ -hydroxy- and  $\beta$ -ketonic acid.



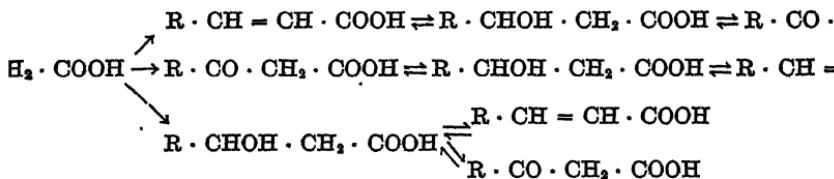
Later, when the oxidation *in vitro* of butyric acid to acetoacetic acid was observed it was also thought at first that  $\beta$ -hydroxybutyric acid was an intermediate product. Further investigation failed to confirm this view and in addition it was found that  $\beta$ -hydroxybutyric acid formed *in vivo* was at any rate partly derived by the reduction of acetoacetic acid. Thus the formation of  $\beta$ -hydroxy-acids as the first stage in the oxidation of saturated fatty acids instead of appearing highly probable became rather problematical. The initial formation of  $\beta$ -ketonic acids appeared more probable.

Subsequently, the unsaturated acids acquired importance in connection with the oxidation of saturated fatty acids. In the first place it was found that unsaturated acids, such as cinnamic acid, could be formed *in vivo* from phenylpropionic acid and related compounds and later on the direct oxidation of succinic to

fumaric acid by Battelli and Stern's "succinoxidon"—a tissue enzyme—was well established. Furthermore, the mutual interconversion of the unsaturated and  $\beta$ -hydroxy-acids was observed in a number of cases both within and without the body. The direct formation of unsaturated acids from saturated ones was intelligible in the light of Wieland's dehydrogenation theory of oxidation.

The literature dealing with the reactions just outlined is extensive and it is not proposed to review it in the present communication. Most of it can be found in the writer's monograph (1) on the subject and also in an article in *Physiological Reviews* (2).

From a consideration of all the facts it would appear that it is possible to advance more or less evidence in favor of regarding either the unsaturated acids, or the  $\beta$ -ketonic acids or, rather less probably, the  $\beta$ -hydroxy-acids, as the initial products of the oxidation of saturated fatty acids.<sup>1</sup> These changes may be represented in the following scheme which also indicates the possible further transformations observed *in vivo* in the case of numerous examples.



It was thought possible that some evidence might be obtained as to the relative probability of one or other of these three types of change being concerned in the initial oxidation of saturated fatty acids by the following line of experiments: Caproic acid was known from Embden's results to give acetoacetic acid and acetone.

<sup>1</sup> Recently, Armstrong (Armstrong, H. E., *J. Soc. Chem. Ind.* 1922, xli, 265) without any apparent regard to existing knowledge has pictured saturated fatty acids as being first oxidized to per acids of the type  $R\text{-CH}_2\text{-CO-O-OH}$  which then undergo rearrangement with formation of  $\beta$ -hydroxy- and  $\beta$ -ketonic acids. So far as reactions *in vivo* are concerned there is apparently no evidence in favor of this idea and much against it. Since Armstrong produces no evidence of any kind to support his views, the reader is left somewhat in doubt whether to take his pronouncements seriously or to regard them as a pleasant *jeu d'esprit*.

or at least a ketone with similar reactions, on perfusion through a surviving liver. The writer's observations have confirmed this and show that the ketone obtained was acetone and not propylmethyl ketone as might possibly be the case. The formation of  $\beta$ -hydroxybutyric acid was also established. The next step was to perfuse through surviving livers under as nearly similar conditions, the unsaturated,  $\beta$ -hydroxy-, and  $\beta$ -ketonic acids derivable from caproic acid. An experiment was also made with the doubly unsaturated sorbic acid. The relation of these acids to each other is shown by the following formulas.

Caproic acid.....	$\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$
$\alpha\beta$ -Hexenic acid .....	$\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH} = \text{CH} \cdot \text{COOH}$
$\beta$ -Hydroxycaproic acid.....	$\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CHOH} \cdot \text{CH}_2 \cdot \text{COOH}$
Butyrylacetic acid.....	$\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{COOH}$
Sorbic acid.....	$\text{CH}_3 \cdot \text{CH} = \text{CH} \cdot \text{CH} = \text{CH} \cdot \text{COOH}$

For the correct interpretation of the results it was necessary to devise methods for the estimation of acetone in the presence of propylmethyl ketone and, as will be seen in the experimental portion of the paper, this was adequately accomplished. With the exception of sorbic acid for which at present there is no good reason for believing it to be a metabolite of caproic acid, it was found that each and all of the remaining acids under similar conditions of perfusion gave rise to large amounts of acetoacetic acid, acetone, and  $\beta$ -hydroxybutyric acid, but that the quantitative differences between the various acids as precursors of "acetone bodies" were insufficient to indicate which, if any, was preferentially produced from caproic acid. The total amount of acetone bodies obtained from the four acids was not widely different. It is therefore not possible to obtain any satisfactory answer from the present experiments as to whether an unsaturated,  $\beta$ -hydroxy-, or  $\beta$ -ketonic acid, is first formed by the oxidation of caproic acid. It would appear more probable that all the acids are in readily shifting equilibrium with each other and are easily interconvertible.

#### EXPERIMENTAL.

##### *Methods.*

The perfusions were made with dog's blood diluted with not more than one-third volume of saline solution. The livers of

medium sized animals of 10 to 14 kilos weight, which were not fed during the day preceding the operation, were employed using the customary technique. The volume of perfusion fluid varied from 1,200 to 1,600 cc. and the concentration of acid added in the form of neutral ammonium or sodium salt was 2 gm. per liter. The perfusion was continued for 60 minutes only. The blood was then collected, measured, and precipitated with 2 volumes of acid mercuric chloride solution in the usual fashion. An aliquot part of the filtrate was then distilled. The distillate was used for the estimation of acetone and other ketones, while the residue was used for the estimation of  $\beta$ -hydroxybutyric acid.

The estimation of acetone presented some difficulties for it was clear that propylmethyl ketone might accompany the acetone in all experiments and would certainly be present in the butyryl-acetic acid perfusions. The first distillate referred to above was redistilled with 2 per cent hydrogen peroxide (15 cc.) and sodium hydroxide (15 cc. of 30 per cent solution). The volume of the second distillate was adjusted to 100 cc. In an aliquot part of this the total ketones were determined by titration with iodine solution in the customary fashion. A separate determination of acetone, excluding propylmethyl ketone, was based on the following observations.

On heating dilute acetone solutions with 7 per cent mercuric sulfate, dissolved in 20 per cent sulfuric acid, the whole of the acetone is precipitated in the form of a white granular precipitate (Denigès ,3). This reaction has been utilized by Oppenheimer (4) for the gravimetric estimation of acetone in urine, working in closed vessels. The writer has found that while propylmethyl ketone readily combines with mercuric sulfate so that it is not recoverable on distillation, the compound formed is much more soluble in dilute sulfuric acid, especially when hot, than the corresponding acetone compound. The following procedure for the determination of acetone in the presence of propylmethyl ketone was found adequate for the purpose in view. A portion of the distillate, obtained as previously described (25 or 50 cc.), is acidified with sulfuric acid (1:1) so that the solution contains 10 per cent sulfuric acid by volume. Mercuric sulfate solution (25 to 50 cc.) is then added and the mixture, contained in a conical flask provided with a reflux tube drawn to a fine point at the end,

is heated for 30 minutes in a rapidly boiling water bath. The precipitate containing the acetone is filtered off while the solution is still hot and washed with water and alcohol. It is then dried in a steam bath. The weight of precipitate multiplied by 0.055 gives the acetone with very fair accuracy. Under the above conditions propylmethyl ketone gives almost no precipitate though some settles out on standing in the cold. A solution of acetone containing 47.5 mg. in 25 cc. according to iodometric analysis gave 47.3 by gravimetric analysis. A similar solution of propylmethyl ketone (100 mg.) gave 0.0075 gm. precipitate, corresponding to 0.4 mg. of acetone. Mixtures of both ketones gave results for acetone of more than sufficient accuracy for the purpose in view.

The formation of  $\beta$ -hydroxybutyric acid as the result of the perfusion of acids, such as caproic acid, through a surviving liver apparently has not been previously investigated though the reduction of acetoacetic acid, which is known to be formed, to  $\beta$ -hydroxybutyric acid is well established as occurring in the liver. In order to gain some idea of the extent of its production use was made of Shaffer's (5) method of estimation, suitably modified for present purposes. The residue left after the distillation of ketones from the blood filtrate was acidified with concentrated sulfuric acid (25 cc.) and then potassium bichromate solution (6 per cent) was added, a few drops at a time as fast as reduced during a rapid distillation. The distillate was then redistilled with hydrogen peroxide and sodium hydroxide as previously described. The second distillate (100 cc.), containing essentially acetone and propylmethyl ketone, was then analyzed (a) iodometrically for total ketones, and (b) gravimetrically for acetone as above described. When the results of the two analyses approximated each other it was inferred that no significant amount of the higher ketone was present.

*Results of Perfusion Experiments.*—In Table I are recorded the results of the various perfusions. In the first column under I the results of the estimations of total volatile ketones in terms of acetone are recorded with the true acetone values as determined gravimetrically. With the exception of the experiments with butyrylacetic acid, in which the unchanged acid gives propylmethyl ketone on distillation, it would appear that no significant

amount of any other ketone than acetone was present. The columns under II contain the results of oxidizing with chromic acid the residue from the first distillation. The total ketone estimations here are probably of very little value, since unchanred  $\alpha\beta$ -hexenic acid and  $\beta$ -hydroxycaproic undoubtedly furnish some propylmethyl ketone on oxidation. The actual acetone determinations made gravimetrically are probably a fair index of the  $\beta$ -hydroxybutyric acid formation and it is interesting to note that the results in many cases are not very much lower than those for the acetone derived from acetoacetic acid.

TABLE I.

Name of acid.	I		II		III Total acetone.	
	Distillate from blood filtrate.		Oxidation with chromic acid residue from I.			
	Total ketones as acetone.	Actual acetone.	Total ketones as acetone.	Actual acetone.		
	mg.	mg.	mg.	mg.	mg.	
Blank.....	12.0	12.0	21.1	20.5	32.5	
Caproic acid.....	80.0	82.1	91.5	87.2	169.3	
	160	172	36.9	31.8	203.8	
$\alpha\beta$ -Hexenic acid.....	131	127	105	54.5	181.5	
	121	113	108	67.8	180.8	
$\beta$ -Hydroxycaproic acid.....	73.5	53.2	952	102	155.2	
	101	103	876	66.5	169.5	
Butyrylacetic acid.....	648	75	285	85.2	160.2	
	766	106	268	78.0	184	
Sorbic acid.....	49.4	50.2	59.1	58.2	108.4	

A few notes on the preparation of the substances used and the results obtained with them are appended.

*Caproic Acid.*—The acid used was a redistilled specimen of the synthetic acid. The results on perfusion indicate a pronounced formation of acetoacetic acid and  $\beta$ -hydroxybutyric acid. The close concordance between the "total ketone" and acetone figures shows that very little, if any,  $\beta$ -hydroxycaproic acid or butyrylacetic acid were in the blood at the close of perfusion, for both of these acids would yield propylmethyl ketone and so increase the figures for total ketone.

$\alpha\beta$ -*Hexenic Acid*.—Caproic acid was brominated with bromine and phosphorus in the usual way and then converted into  $\alpha$ -bromo-caproic ester by treatment with alcohol. The ester was boiled with diethyl aniline according to the directions of Blaise and Luttringer (6) in order to remove hydrobromic acid. The resulting ester was hydrolyzed and the free acid carefully fractionated. The product used was entirely free from bromine compounds.

The results on perfusion indicate a strong acetoacetic acid formation unaccompanied by butyrylacetic acid. The acetone derived from  $\beta$ -hydroxybutyric acid is also considerable in amount. The excess of total ketones over acetone on chromic acid oxidation may well be assumed to be due to the formation of some  $\beta$ -hydroxy-caproic acid during perfusion.

$\beta$ -*Hydroxycaproic Acid*.—Two preparations of this acid were used. One was made according to Fittig and Baker's method (7) from  $\beta$ -bromocaproic acid which in turn was prepared from  $\alpha\beta$ -hexenic acid. The other preparation was prepared by the following rather more convenient method. Butyrylacetic ester (20 gm.), prepared as described in the next section, dissolved in alcohol (20 cc.), was mixed with water (180 cc.) and the whole cooled in an ice bath. Sodium amalgam (200 gm. of 4 per cent) was added fairly rapidly with good mechanical stirring. Less than a gram of oil was left undissolved and this was removed the following day by extraction with ether. Nine-tenths of the solution were made just acid with sulfuric acid (1:1), then the remaining one-tenth was added and the whole concentrated on a water bath. On making the residue just acid to Congo red with sulfuric acid the oily acid partly separated and was extracted with ether. On evaporation of the ether extracts and drying *in vacuo*, 10 gm. of acid remained which appeared to be identical in all respects with Fittig and Baker's product.

The perfusion results showed that the acid gave rise to both acetoacetic and  $\beta$ -hydroxybutyric acid in marked degree. In one case the difference between total ketone and acetone in the blood filtrate distillate indicated a possible butyrylacetic acid production. On oxidation with chromic acid much propylmethyl ketone was naturally formed thus giving a high total ketone figure, but acetone derived from  $\beta$ -hydroxybutyric acid was undoubtedly present in fair amount.

*Butyrylacetic Acid.*—The ethyl ester of this acid was prepared by the condensation of butyric and acetic esters by means of sodium as outlined by Wahl and Doll (8). The details of the apparatus and mode of operation were the same as those used by Dakin and Dudley (9) for the preparation of ethyl  $\gamma$ -diethoxyacetacetate. The proportions used were the following: ethyl butyrate 232 gm., ethyl acetate 176 gm., and sodium 46 gm. The yield of crude ester was 76 gm. of oil, boiling from 80–105° at 15 mm. pressure. The crude product was purified by repeated shaking with sodium bisulfite solution with which it does not combine, and then refractionated. The main, middle fraction, boiling steadily at 87–89° at 8 to 10 mm. pressure, was collected and used for the preparation of the acid. The hydrolysis of the ester was effected in the cold with sodium hydroxide and the product worked up as is customary with acetoacetic acid. The dilute solution of the sodium salt was well boiled at room temperature under greatly diminished pressure in order to remove traces of alcohol or ether. Its concentration was determined by distilling a portion of the solution and estimating the propylmethyl ketone in the distillate with standard iodine solution. The results of the perfusion indicate a considerable production of both acetoacetic acid and  $\beta$ -hydroxybutyric acid. The excess of total ketones obtained by chromic acid oxidation compared with the actual acetone concentration appears to indicate a considerable reduction of the  $\beta$ -ketonic acid to  $\beta$ -hydroxycaproic acid. This change is, of course, analogous to the known reduction of acetoacetic acid to  $\beta$ -hydroxybutyric acid.

*Sorbic Acid.*—The substance was obtained by the condensation of crotonic aldehyde and malonic acid with pyridine according to Doebner's (10) method. The perfusion results indicate that the acid, while definitely giving some acetoacetic acid and  $\beta$ -hydroxybutyric acid, is much less effective in this respect than the other acids examined. There would seem no good reason to regard it as a probable normal metabolite of caproic acid.

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ON THE ENDOOTHERMIC REACTION WHICH ACCOMPANIES THE APPEARANCE OF A VISIBLE CURD IN MILKS COAGULATED BY HEAT: A CONTRIBUTION TO THE THEORY OF THE HEAT COAGULATION OF MILK.

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It is an accepted fact that the casein of milk, in part at least, is in true colloidal suspension. In the colloidal state the casein is subject to precipitation or coagulation by heat as are numerous other colloids. There is no definite temperature of coagulation with casein or other proteins. There is rather a time-temperature relationship. In other words, milk must be subjected to the higher temperatures for certain periods of time before coagulation occurs. As would be expected, the higher the temperature, the shorter is the time required for precipitation to start. The principal factors that affect the time-temperature relationship in milk appear to be acidity, protein concentration, and the salt balance. In the case of evaporated milk the time-temperature requirements for the complete sterilization of the finished product are very close to those for coagulation. It is, therefore, quite essential that the sterilization process be carried out with great care, or spoilage of the material results. In condensed milk thickening of the product often occurs after weeks or months of storage at ordinary temperatures. Since it will be shown in this paper, among other things, that the mechanism of the thickening of milks concentrated at low temperatures is essentially the same as that which results in coagulation when heat is applied to the product, we shall use the term heat coagulation as designating both effects.

It is the purpose of this article to present certain facts concerning the heat coagulation of milk which bring us nearer to the

point where an adequate theory of coagulation may be formulated. While it will be apparent to the reader that the work described in this paper has not as yet been completed, a number of very definite relationships are pointed out which should be of considerable scientific importance to others working upon the proteins, and which will perhaps be of interest also to the evaporated and condensed milk industries. The authors believe that this justifies them in reporting upon the work which they have done to date, leaving future developments to another paper.

This paper will show that the process of heat coagulation is accompanied by an endothermic chemical reaction; that this reaction is undoubtedly the precipitation of calcium and magnesium; that the main reaction concerns also the metals in combination with the protein of the milk; and further that the thickening of sterile, highly concentrated milks, both sweetened and unsweetened, upon standing at room temperature, is a similar reaction instead of a bacteriological effect as heretofore believed. Other significant effects of the preliminary forewarming of milk upon this endothermic reaction are shown, together with the effect upon the stability of the milks toward heat.

Before reporting upon the present experimental work, it will be well to summarize, from the literature, previous important work upon the heat coagulation of the proteins, in order that some of the pertinent facts may be correlated with the observations made. In studying the literature dealing with the reactions which cause the precipitation of proteins from suspension or solution upon the application of heat, we are impressed by the diversity of opinion regarding the nature of these reactions, also by the fragmentary character of the individual researches. This is especially true of those papers dealing with the heat coagulation of the proteins of milk.

Chick and Martin (1) have shown, they believe, that the heat coagulation of egg albumin and hemoglobin is a reaction between protein and water, and that temperature merely has an accelerating action. The reaction has an extraordinarily high temperature coefficient, and the velocity is markedly influenced by a variety of conditions, the most important of which is the presence of acid. Chick and Martin (2) have further shown that the heat coagulation of proteins consists of two processes—denaturation and agglutination.

Robertson (3) gives perhaps the best summary of all the work done on

proteins. He points out that the heating of proteins in solution probably results not only in the abstraction of the elements of water from the protein, but also in the polymerization of the protein molecule. He calls attention to the work of Pauli and coworkers (4) and Pascheles (5) on the influence of a variety of salts in acid and alkaline solution upon the coagulation of proteins, and cites Pauli's results as supporting the theory that heat coagulation is accomplished through the dehydration of the protein molecules. He summarizes the work of Chick and Martin (1, 2) above referred to and points out that, while Chick and Martin believe coagulation is brought about by hydration, he believes that his own work and that of Pauli show the nature of the action to be one of dehydration, basing their conclusion on the fact that the base-combining capacities of the proteins diminish with heat. The theory that coagulation takes place in two stages, as outlined by Chick and Martin, receives his support.

If we come now more particularly to the work that has been done upon milk, we must not pass over two articles in the French literature which deal with the rennet precipitation of the milk. Van Dam (6) claims to show how the time of coagulation is influenced by the soluble calcium salts. He advances the revolutionary idea that the calcium salts of the serum have little effect upon determining this time, but that the calcium combined with the casein plays the important part in the coagulation process. According to him, milks high in lime are more easily coagulated than others.

Lindet (7) also has published a most interesting paper dealing with this subject. He states that the albumin of milk is a casein differing from normal casein only in its rotary power. After showing that these two caseins are solubilized by the presence of the mineral salts of the milk he continues:

I have shown that the caseins are dissolved by the elements of the serum and especially by the phosphates and the alkaline citrates; on the other hand, I have recognized that the solubilized caseins solubilize themselves by their acid function, from the lime, the calcium phosphates, the magnesium, etc., and that this mineralization aids them to dissolve. If now we introduce calcium chloride into this mixture the alkaline phosphates and citrates will give calcium phosphate and calcium citrate; . . . . the calcium phosphate being substituted for sodium phosphate, the soluble caseins lose one of their solvents. At the same time the caseins which were dissolved in sodium citrate are found in the presence of calcium citrate, in which they are insoluble. It is not then surprising that a part of the soluble caseins retrograde, since their solvents disappear. . . . There is another reaction added to the preceding. The bicalcic-phosphate produced is not stable. It is subject to dissociation in the presence of water and decomposes into tricalcic-phosphate and acid-monocalcic-phosphate; further, a continuous reaction is produced. The acid phosphate attacks the lime of the soluble casein, forms again bicalcic-phosphate, which will in turn be decomposed, and so on until the caseins are deprived of the lime which facilitates their solution.

Milroy (8) has shown that fresh milk which has been subjected to a temperature slightly below boiling for 1 hour shows an increase in acidity

and a fall in the soluble calcium content. This milk is only slowly acted upon by rennet. The coagulability of heated milk may be raised either by adding calcium chloride or by otherwise increasing the acidity. The former does not act simply by raising the acidity; nor does the increase of acidity act simply by affecting the soluble calcium content.

We come now to one of the most interesting pieces of work from our point of view—the paper by Sommer and Hart (9). The main points of this article are that there is an optimal ratio between the calcium salts and the protein in milk which tends for stability of the protein; also that the hydrogen ion concentration is not the determining factor regarding stability. The removal of calcium prevents coagulation in most cases and, similarly, in most cases the addition of small amounts of calcium salts lowers the coagulating point. This coagulation can again be balanced by means of sodium citrate or dipotassium phosphate. It is also stated that the danger of coagulation may be avoided in the actual practice of condensing milk by lengthening the preheating period, using higher temperatures. This may have the effect of precipitating soluble calcium.

Schryver (10) gives an account of the action of calcium salts on sodium cholate. It was found that in the case of those salts which raise the surface tension of water, the greater the concentration of the salt, the shorter was the time required for clot formation. In the case of salts which lower the surface tension, on the other hand, increase of concentration decreased the clotting time only up to a certain limit of optimal concentration. Above this limit the clotting time was diminished, or the clot formation inhibited entirely. Somewhat similar results were obtained upon caseinogen. The statement is also made that evidence points to the fact that the clot is formed from free caseinogen or *m*-caseinogen and not from the calcium salt.

From the foregoing papers we see that the heat coagulation of milk is a complex process. There are numerous factors that come into play, and until these are all taken into consideration no satisfactory theory can be developed. Chick and Martin, and Robertson have shown the possibility of the denaturation of the protein molecule and of the hydration or dehydration reactions, while the other authors quoted have shown to a certain extent the effects of the mineral constituents of milk upon its stability. Their work suggests that reactions involving these mineral constituents may be brought about by heat. Bearing these facts in mind, we can now proceed to the experimental work to be reported here.

#### EXPERIMENTAL.

This work has all been done with skim milk and skim milk products unless otherwise stated. The reason for this is that the

presence of fat influences the heat coagulation of milk and interferes with the heat measurements to be described. Data showing the effect of fat will be given later. As is customary, the term evaporated milk in this paper means a concentrated milk, while the term condensed milk means a concentrated milk containing sucrose.

We will first point out that if a normal skim milk or an evaporated skim milk be heated until coagulation takes place, the usual result is a *marked heat absorption coincident with the appearance of a visible curd*. The existence of this endothermic reaction was shown during the course of some work in which the rate of heating of milk in an autoclave of the size of an ordinary condensed milk can was determined, when the cool autoclave was immersed in a high temperature oil bath. The procedure will be described in detail, since a method based upon this heat absorption has been employed to gauge the relative stability of different milks toward heat.

A steel autoclave approximately the size of a small condensed milk can is employed. As constructed, the cover shuts upon a rubber gasket and can be tightly closed by means of four bolts. The temperature of the milk within the autoclave can be measured by means of either a thermocouple or a thermometer. If a thermometer is to be used, it must be inserted through a stuffing-box. The design given by Magoon and Culpepper (11) seems to be the best for this purpose.

If a thermocouple is to be used, a steel well, the walls of which are about  $\frac{1}{8}$  of an inch thick may replace the stuffing-box. The hot junction of the thermocouple is inserted into this well, which projects into the center of the autoclave. With this method there are perhaps errors of conduction, etc., but since all temperature measurements are comparative, these errors can be neglected for the purpose of this work.

A five-junction thermocouple made of copper and constantan No. 36 wire was employed for the work herein described. The junctions were encased in fine glass tubes of sufficient length to prevent any of the wire from coming into actual contact with the walls of the autoclave well. This precaution was taken since an electrically heated oil bath was used for heating, and in case the silk insulation of the wire were to become wet with oil, at the temperatures employed, the wet insulation would be sufficiently

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conducting to cause trouble with the voltage measurements. The cold junction was kept at  $0^{\circ}$  in an ice water bath. The thermocouple voltage was measured by means of a Leeds and Northrup Type K potentiometer. By this method the temperature of the autoclave contents could be measured within approximately  $\frac{1}{200}$  of a degree.

A suitable high temperature oil bath completed the equipment. The temperature of this bath was maintained automatically to within  $\pm 0.025^{\circ}$ .

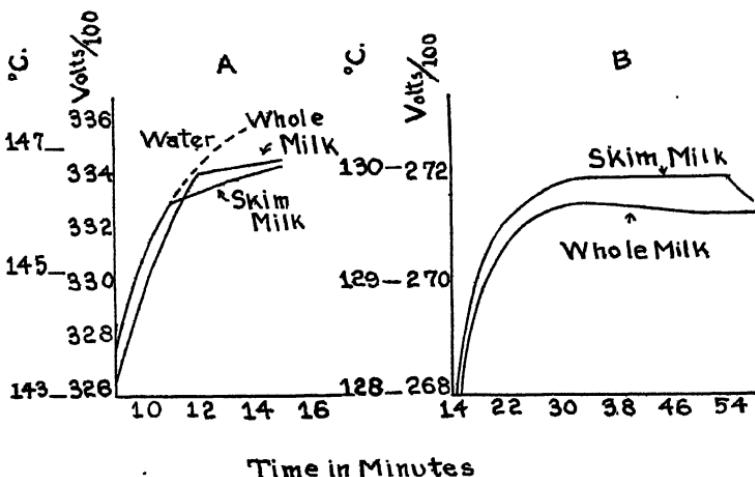


Fig. 1, A. Heat penetration curves of skim and whole milks which coagulated before the temperature of the oil bath was reached.

Fig. 1, B. Heat penetration curves of skim and whole milks which coagulated after the temperature of the oil bath was reached.

Now, to return to the heat absorption phenomenon. The endothermic nature of the coagulation reaction may be demonstrated in one of two ways. For the time being we are considering only skim milk. We may choose a temperature in the oil bath sufficiently high so that, if the autoclave containing milk be immersed in it, the milk will coagulate before the temperature of the bath is reached. If we measure the temperature within the autoclave periodically, let us say every minute, we may plot the data on a curve sheet, plotting time and temperature. The

upper part of such a curve is given in Fig. 1, A. It will be noticed that there is a distinct flattening of the curve. Repeated tests have shown that the flattening of the curve takes place coincidentally with coagulation. This flattening is more apparent if we also plot, beside this curve, a curve representing temperatures attained within the autoclave under similar conditions when water is present instead of milk. Or, we can choose a bath temperature sufficiently low so that the milk will have time to reach the temperature of the oil bath before coagulation takes place. In this case, Fig. 1, B, we note that there is a slight temperature drop which occurs coincidentally with the appearance of a visible curd. We have then two modifications of a general method of determining the precise moment that coagulation takes place when a milk is coagulated by the influence of heat. The coincidence of the heat absorption and the appearance of a visible curd in the milk has been demonstrated repeatedly throughout tests covering daily experiments for more than a year. In no case has there been an absorption in skim milk without coagulation. Except as herein noted there was no coagulation without some detectable heat absorption.

For the evaporated milk industry, where it is necessary that a determination be made of the amount of heat that a milk will stand during sterilization, without producing a curd which cannot be shaken out, it seemed at first as if a method could be worked out, based on the above, which would do away with the use of the pilot sterilizer.

In a series of daily examinations of skim milk from the Dairy Division Farm at Beltsville, however, it has been shown that there is a wide variation in the magnitude of the temperature drop obtained by the second method outlined. This work was all done upon normal skim milk. A temperature of  $135^{\circ}$  was used as the highest it was possible to employ while still making certain that the break would occur upon the flat of the time-temperature curve. Temperature drops of a magnitude of from 0.05 to  $1.50^{\circ}$  were obtained. In only two or three instances could *no* heat absorption be detected. These instances all occurred with milks that were particularly resistant to the action of heat. The same irregularities appear in evaporated milks. Since the temperature drop was usually so slight, a majority of the milks giving only from 0.05 to

0.10,<sup>°</sup> we must discard this as a useful method until further work may perhaps enable us to magnify the amount of the heat absorption. On the other hand, in no instance have we failed to obtain a break in the time-temperature curve when the milk coagulated during the time in which its temperature was rising. The first method outlined is, therefore, a valuable laboratory method for determining roughly the relative stabilities of different milk samples toward heat, though experiments have not as yet shown that it parallels the commercial pilot sterilizer test closely enough to make it seem of any particular value to the industry.

Before going further, we must call attention to the effect of the presence of fat upon these curves. If fat is present there is apparently a *continuous heat absorption* during heating. This usually serves to mask the "drop" of the second method entirely, but does not interfere with the first method. Figs. 1, A and 1, B.

We have established one fact, therefore; namely, that an endothermic reaction takes place coincidentally with the heat coagulation of the casein of milk. We proceed now to those observations bearing upon the thickening of condensed milk upon prolonged standing at normal temperatures. This thickening has been considered to be a result of bacteriological action. One of us studying the matter from this bacteriological point of view, found, as will be shown, that the reaction was chemical and physical, not bacteriological. In the course of the investigation it became necessary to prepare sterile milk for control purposes. The milk sterilized for 10 minutes in the autoclave at 120<sup>°</sup> before condensing never thickened after it was condensed and put in storage. A similar milk that had been forewarmed at 95<sup>°</sup> thickened readily. An unforewarmed condensed milk did not thicken.<sup>1</sup>

The significance of these latter observations will be pointed out further on in the paper.

While trying to determine the temperature at which thickening would take place in order to develop an accelerated aging test, the observation also was made that the thickening reaction was accompanied by a marked heat absorption. Upon making up an artificial milk serum, such as was employed by Palmer (12) in

<sup>1</sup> This observation is in accord with observations made in these laboratories and recorded by Rogers, Deysher, and Evans (Rogers, L. A., Deysher, E. F., and Evans, F. R., *J. Dairy Sc.*, 1920, iii, 469).

his work on lipase, it was further shown that a corresponding heat absorption could be obtained from this serum. Coincident with this absorption a white precipitate was formed.

The artificial serum, as we will show, contains calcium, magnesium, sodium, and potassium cations, together with phosphate, citrate, and chlorine anions. In addition there are present the sugars, lactose and sucrose. The precipitate obtained by heating this mixture must evidently contain calcium, magnesium, or both these metals, together with some of the anions present in the mixture. Rough heat absorption tests were made to determine the reaction which would account for the heat absorption. The results in Table I were obtained with variations of the normal serum.

TABLE I.  
*Effect of Serum Composition on Heat Absorption.*

Serum.	Heat absorption.
Made up without lactose.	Normal.
" " " sucrose.	"
" " " calcium.	Very slight.
" " " magnesium.	Nearly normal.
" " " calcium or magnesium.	None.
" " " citrates.	Less than normal.
" " " phosphates.	" " "

In other words, the heat absorption comes from the precipitation of calcium and magnesium as phosphates and citrates.

From the above we may conclude that the thickening of condensed milk under the influence of heat is accompanied by an endothermic chemical reaction, which results in the precipitation of at least a portion of the metals present.

It may be pointed out here that the artificial serum as prepared by Palmer is more acid than normal milk. If the tribasic citrates and phosphates are employed as given by Van Slyke and Bosworth (13) a more normal serum is obtained than that given by Palmer, who used acid citrates and phosphates.

We may now prove one more point of considerable theoretical interest. In Fig. 2, A, we give the heat absorption curve obtained on an artificial serum, as compared with a normal water curve.

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0.10,<sup>°</sup> we must discard this as a useful method until further work may perhaps enable us to magnify the amount of the heat absorption. On the other hand, in no instance have we failed to obtain a break in the time-temperature curve when the milk coagulated during the time in which its temperature was rising. The first method outlined is, therefore, a valuable laboratory method for determining roughly the relative stabilities of different milk samples toward heat, though experiments have not as yet shown that it parallels the commercial pilot sterilizer test closely enough to make it seem of any particular value to the industry.

Before going further, we must call attention to the effect of the presence of fat upon these curves. If fat is present there is apparently a *continuous heat absorption* during heating. This usually serves to mask the "drop" of the second method entirely, but does not interfere with the first method. Figs. 1, A and 1, B.

We have established one fact, therefore; namely, that an endothermic reaction takes place coincidentally with the heat coagulation of the casein of milk. We proceed now to those observations bearing upon the thickening of condensed milk upon prolonged standing at normal temperatures. This thickening has been considered to be a result of bacteriological action. One of us studying the matter from this bacteriological point of view, found, as will be shown, that the reaction was chemical and physical, not bacteriological. In the course of the investigation it became necessary to prepare sterile milk for control purposes. The milk sterilized for 10 minutes in the autoclave at 120<sup>°</sup> before condensing never thickened after it was condensed and put in storage. A similar milk that had been forewarmed at 95<sup>°</sup> thickened readily. An unforewarmed condensed milk did not thicken.<sup>1</sup>

The significance of these latter observations will be pointed out further on in the paper.

While trying to determine the temperature at which thickening would take place in order to develop an accelerated aging test, the observation also was made that the thickening reaction was accompanied by a marked heat absorption. Upon making up an artificial milk serum, such as was employed by Palmer (12) in

<sup>1</sup> This observation is in accord with observations made in these laboratories and recorded by Rogers, Deysher, and Evans (Rogers, L. A., Deysher, E. F., and Evans, F. R., *J. Dairy Sc.*, 1920, iii, 469).

his work on lipase, it was further shown that a corresponding heat absorption could be obtained from this serum. Coincident with this absorption a white precipitate was formed.

The artificial serum, as we will show, contains calcium, magnesium, sodium, and potassium cations, together with phosphate, citrate, and chlorine anions. In addition there are present the sugars, lactose and sucrose. The precipitate obtained by heating this mixture must evidently contain calcium, magnesium, or both these metals, together with some of the anions present in the mixture. Rough heat absorption tests were made to determine the reaction which would account for the heat absorption. The results in Table I were obtained with variations of the normal serum.

TABLE I.  
*Effect of Serum Composition on Heat Absorption.*

Serum.	Heat absorption.
Made up without lactose.	Normal.
" " " sucrose.	"
" " " calcium.	Very slight.
" " " magnesium.	Nearly normal.
" " " calcium or magnesium.	None.
" " " citrates.	Less than normal.
" " " phosphates.	" " "

In other words, the heat absorption comes from the precipitation of calcium and magnesium as phosphates and citrates.

From the above we may conclude that the thickening of condensed milk under the influence of heat is accompanied by an endothermic chemical reaction, which results in the precipitation of at least a portion of the metals present.

It may be pointed out here that the artificial serum as prepared by Palmer is more acid than normal milk. If the tribasic citrates and phosphates are employed as given by Van Slyke and Bosworth (13) a more normal serum is obtained than that given by Palmer, who used acid citrates and phosphates.

We may now prove one more point of considerable theoretical interest. In Fig. 2, A, we give the heat absorption curve obtained on an artificial serum, as compared with a normal water curve.

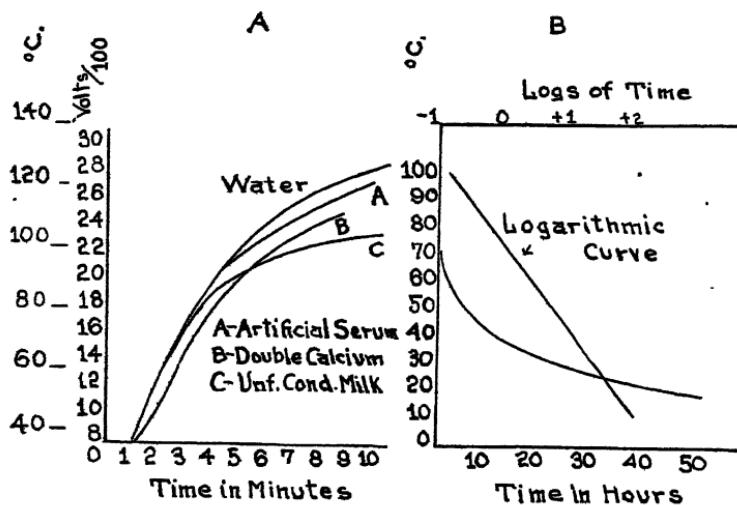


FIG. 2, A. Heat absorption curves of artificial serum, unforewarmed condensed milk, and a serum containing an amount of calcium equivalent to the total calcium of normal milk.

FIG. 2, B. Time required to produce coagulation in 95° forewarmed condensed milk samples stored at different temperatures.

There is also given the heat absorption curve of a normal condensed skim milk. The condensed milk has the composition:

	per cent
Milk solids (no fat is present).....	20
Cane-sugar.....	48
Water.....	32
Total.....	<hr/> 100

The artificial serum was made up to have the same salt concentration as the serum of the condensed milk. The weights used were:

	gm.
CaCl <sub>2</sub> .....	0.476
MgH <sub>4</sub> (PO <sub>4</sub> ) <sub>2</sub> .....	0.412
K <sub>2</sub> HPO <sub>4</sub> .....	0.920
Na <sub>3</sub> C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> .....	0.888
K <sub>3</sub> C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> .....	0.208
CaHPO <sub>4</sub> .....	0.730
Lactose.....	19.60
Cane-sugar.....	86.48
Water.....	57.65

This serum is based upon Van Slyke's formula for the individual compounds present in milk. It is as follows:

	<i>per cent</i>
Fat.....	3.900
Lactose.....	4.900
Proteins, combined with calcium (1.78 per cent of the protein is calcium).....	3.200
Dicalcium phosphate.....	0.175
Calcium chloride.....	0.119
Monomagnesium phosphate.....	0.103
Sodium citrate.....	0.222
Potassium citrate.....	0.052
Dipotassium phosphate.....	0.230
 Total.....	12.901
Water.....	87.099

The point which at once becomes apparent upon studying these curves is that the heat absorption obtained with the condensed milk is markedly greater than that obtained with the serum. Now Van Slyke and Bosworth (14) point out that 1.78 per cent of the protein present in normal milk is calcium. In our artificial serum we have used an amount of calcium equivalent only to that present in the serum. Suppose we now add calcium chloride to our serum to increase its calcium content to that present in normal milk (serum calcium plus protein calcium) and run a heat absorption curve. The magnitude of the heat absorption is fully commensurate with that obtained with condensed milk. This means, then, that *the combined calcium is a factor in the coagulation of milk.* Another point of interest in the curves is that precipitation of the calcium starts immediately when the high calcium serum begins to warm up, and, if we are to judge from the slope of the heating curve, the reaction is very rapid, much more so than in the case of condensed milk. The rate of *precipitation* of condensed milk calcium must then *be governed, to a certain extent at least, by the reaction rate of the calcium of the protein.* This is an important theoretical point, since from the above it is apparent that conditions favoring the reaction of the calcium of the protein must be those which cause instability of the milk.

In the experiments with artificial serum it was apparent that, while there was no difference in the heat absorption curves when cane-sugar was present, or absent, there was a marked difference

in the nature and volume of the precipitate obtained. The precipitate apparently was soluble to some extent in the sucrose. It was also more gelatinous when the sugar was present.

Thus far we have assumed from evidence at hand that the mechanism of the thickening reaction of milk taking place at the ordinary temperatures is the same as that of the coagulating reaction at the higher temperatures. To get some information directly upon this point, a number of cans of 95° forewarmed condensed milk were placed in constant temperature ovens at temperatures of 20, 30, 40, and 57°C. The viscosity of the original sample at 20° was such that the viscosimeter employed showed an angular deflection of 12°. The viscosities of the samples in storage were measured every hour, a fresh can being used in each

TABLE II.  
*Effect of Storage Temperature on the Time Milk Remains Unthickened.*

Temperature of storage.	Time required to start thickening.
°C.	hrs.*
57	3½
40	11½
30	23½
20	47½

\*  $\frac{1}{2}$  hour was the allowance made for oven temperature to be reached.

determination. If we record the time required at each temperature for the milk to reach a viscosity such that the angular deflection of our viscosimeter is 18° at a temperature of 20° we will record very closely the time required for the thickening reaction to start at the different temperatures (Table II).

The data of Table II are plotted in Fig. 2, B. A smooth curve results. If these data are plotted logarithmically, using the logarithms of time against temperature, we get an approximately straight line. This is taken as a definite proof that the reaction at the lower and higher temperatures is identical. If we extend the logarithmic curve to 95° we see that the condensed milk should from our curve coagulate in 7½ minutes. This is by no means an unreasonable figure.

We shall now investigate the effects of forewarming on the stability of condensed and evaporated milks. It will also be of

interest to investigate the effect of the cane-sugar. Dr. Geo. E. Holm and Mr. E. F. Deysher of the Dairy Division Laboratories in an unpublished report have shown that usually the forewarming of a normal milk tends to make it less stable to the action of heat, but if the milk is concentrated after having been forewarmed to about 95° for 10 minutes, apparently a point is always reached when in the concentrated milk the forewarmed product becomes more stable than the unforewarmed. It is of considerable interest to note that the forewarmed and unforewarmed milks have equal stability at a concentration of about 12 to 13 per cent milk solids.

The conclusion is drawn, therefore, that while heating milk tends to make it less stable to subsequent heating, the forewarming of an evaporated milk makes the finished product more stable in relation to heat. The effect of the degree of concentration on the stability of condensed milk has not yet been worked out; however, as previously stated, the methods of heat treatment which stabilize evaporated milk render the sweetened product less stable. The converse appears to be true also; namely, that those treatments which unstabilize evaporated milk stabilize condensed milk.

It has been the practice in these laboratories when making evaporated and condensed milks to forewarm the raw milk on a steam bath at a temperature of 95° for 10 minutes. In the factories the forewarming is usually carried out by turning live steam into the hot wells. The results to follow have been obtained with a 10 minute forewarming at three temperatures—95, 110, and 120°. The higher temperature forewarmings were carried out in an autoclave. It is apparent that the work cannot be considered complete until the effects of continued periods of forewarming at different temperatures have been studied. The stabilities of the different milk samples were determined by heat absorption measurements. The data from only one original sample of milk are considered, since it is typical of results which have been obtained repeatedly.

Samples of the skim milk were taken, the proper amount of sugar was added, and while one portion was not forewarmed, the others were forewarmed at 95, 110, and 120°. After the forewarming, all the samples were concentrated by evaporation under reduced pressure till a percentage of 20 per cent milk-solids-not-

fat was obtained with 48 per cent sugar in the finished product. The stabilities of these samples of milk toward heat showed the effect of temperature of forewarming on normal condensed milk. Three other samples of the same milk were then taken and forewarmed at the different temperatures without the addition of sugar. The sugar was added before evaporation, however. Any difference in the final milks, obtained in this way, as compared with the normal condensed milk samples, would be due to the action that the cane-sugar might have during forewarming.

Next, double sized samples of unforewarmed and forewarmed milks were concentrated without the addition of sugar to a point such that if sugar were added they would have the concentration of normal condensed milk. This gave a series of evaporated milks equivalent in concentration to the condensed milks. These portions of evaporated milk were divided, and to one set sugar was added in the correct amount. This series then represented condensed milk in which the sugar was not present during forewarming and concentration. The differences in the properties of this series and the second series would be due to the effect of the sugar during concentration. The differences between this third series and the evaporated milks would be that of the presence of sugar during the coagulating process only.

These milk samples were in turn placed in the autoclave herein described and were plunged into an oil bath whose temperature was 135°C. There was no particular reason for taking just this temperature, but it is sufficient to cause rapid curdling. The temperature within the autoclave was determined every 30 seconds for a period of 10 to 12 minutes. On plotting the temperatures in the autoclave against time and comparing with a normal water curve, it became evident at once that there was considerable heat absorption and that this absorption varied considerably with the different samples. The coagulation was so quickly started under these conditions that it was difficult to determine the instant that coagulation began. Since, however, it is apparent that the more unstable the milk, the greater the reaction velocity, and the greater the rate of heat absorption, the relative positions of the curves give us our information. The curves obtained with the four series of milks are given in Fig. 3.

Since the positions of the curves indicate the relative stabilities of the different milks, we may take some arbitrary time, say 9 minutes, and record the temperatures attained by the different

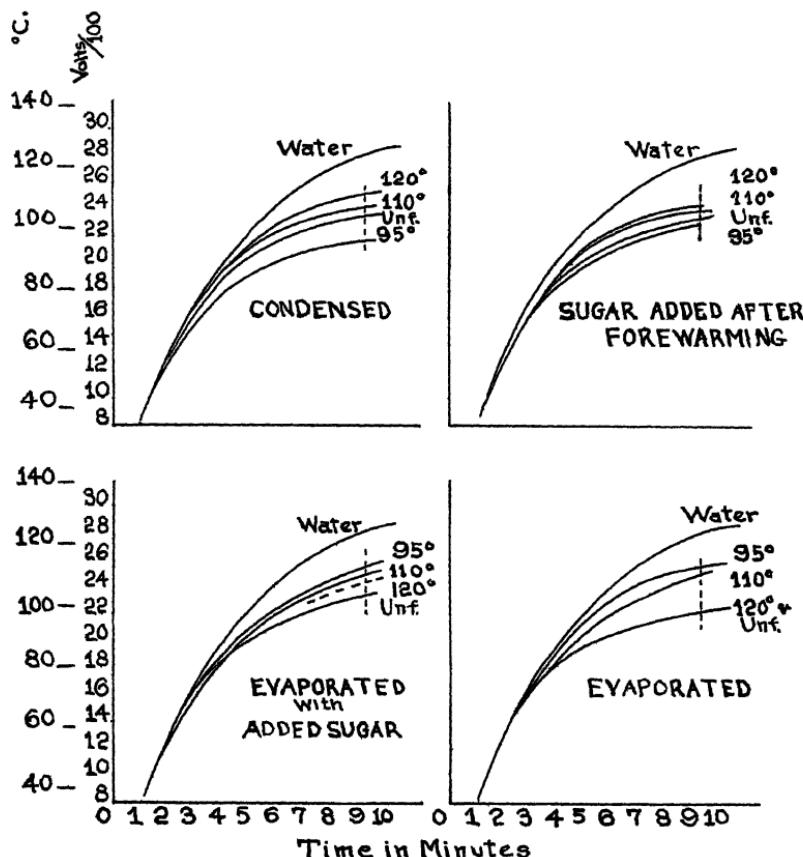


FIG. 3. Heat penetration of stability curves of condensed milks forewarmed at different temperatures, of condensed milks to which sugar was added after forewarming, of evaporated milks to which sugar has been added, and of evaporated milks.

milks in that time. We then have a series of values which we may term *stability figures*. For these we have taken the thermocouple voltage readings without taking the time to convert them to

temperature readings by dividing by 228. Since the potentiometer can be read to five decimal places, it is evident that the third figure of the value is quite significant. A table can be made up of these values which roughly expresses all relationships (Table III).

If we now plot the data for the different milks in Fig. 4, A, plotting temperature of forewarming against stability, and using room temperature, 30°C., as the temperature of the heat treatment of the unforewarmed or normal sample, we see that the condensed milk is first unstabilized, then stabilized, by forewarming, while the evaporated milk, on the contrary, is first more stable, then less so. The curves for the milks to which the sugar was added after forewarming, and for those in which the sugar was added to the evaporated milks, are intermediate.

TABLE III.  
*Stability Figures.*

Condition.	No. 1. Sugar added before forewarming and before concentrating.	No. 2. Sugar added after forewarming but before concentrating.	No. 3. Sugar added after both forewarming and concentrating.	No. 4. Evaporated milk, no sugar.
Unforewarmed . . . . .	235	235	235	222
Forewarmed at 95°..	218	231	255	255
" " 110°..	242	242	248	251
" " 120°..	252	244	244	222

There is one more relationship that is perhaps even more interesting. If we plot the differences in the stability figures of the different columns, we obtain data (Table IV) and curves which show the effect of sugar during forewarming, during concentration, and after evaporation. These curves are given in Fig. 4, B.

The difference in the values between Columns 1 and 2, in Tables III and IV, shows the effect of sugar during forewarming; the difference between Columns 2 and 3 shows the effect of sugar during evaporation; and the difference between the last two shows the effect of the presence of sugar on normal evaporated milks. The results are somewhat of a surprise. While the data in Table IV seem to be irregular, the curves plotted in the above manner are quite regular and nearly identical in shape. We must conclude from them that the action of the sugar is specific and that its effect is superimposed, as it were, upon the other reactions produced by heat.

TABLE IV.

*Action of Sugar during the Various Processes of Milk Condensing under Different Temperatures of Forewarming.*

Condition.	No. 1, Table III. Condensed milk.	No. 2, Table III. Forewarmed, then sugar added.	Curve 1, Fig. 4, B. Effect of forewarming, No. 1 minus No. 2.	No. 3, Table III. Sugar added after evaporation and concentration.	Curve 2, Fig. 4, B. Effect of evaporation, No. 2 minus No. 3.	No. 4, Table III. Evaporated milk, no sugar.	Curve 3, Fig. 4, B. Effect of sugar during coagulation, No. 3 minus No. 4.
Unforewarmed.....	235	235	0	235	0	222	.13
Forewarmed at 95°.....	218	231	-13	255	-24	255	0
"    " 110°.....	242	242	0	248	-6	251	-3
"    " 120°.....	252	244	8	244	0	222	22

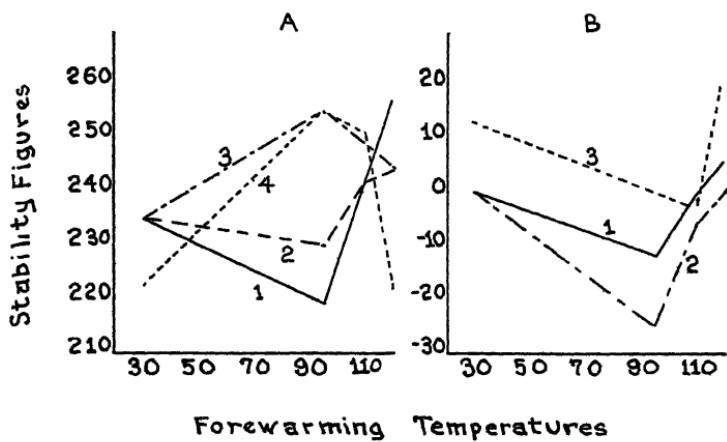


FIG. 4, A. Curves showing the relative stability of condensed milk, Curve 1; condensed milks with sugar added, after forewarming, Curve 2; evaporated milks with added sugar, Curve 3; and plain evaporated milks, Curve 4.

FIG. 4, B. Curves showing the effect of sugar during the various processes of milk condensing, under different temperatures of forewarming. Effect of sugar during forewarming, Curve 1. Effect of sugar during concentration, Curve 2. Effect of sugar during the heat treatment which results in coagulation, Curve 3.

The temperature-time curves given here have been used to gauge the relative stability of different milk samples subjected to comparatively high temperatures. A few preliminary tests of milk samples similar to these seem to indicate that the above relationships *do not* strictly hold at the lower temperatures. The relative stability at 50°C. was ascertained by sealing the milks in test-tubes and placing them in an oven heated to that temperature. Sufficient work has not been done for definite conclusions, but all the data seem to indicate that while the general relationships at the higher temperatures hold, there are two variations. The first is that the evaporated milks (which are undiluted with sugar) coagulate at 50° much more rapidly than do the condensed milks; and the second is that, apparently, the highly forewarmed condensed milks actually are less stable than the heat absorption curves would indicate them to be.

It would perhaps be expected that the heat absorption measurements of highly forewarmed condensed milks might be in error, since it is our opinion that sugar accelerates the removal of calcium from an active state under the conditions of prolonged forewarming. This would mean that there is in the finished product less calcium to react under the influence of heat, and that as a result the position of the heat absorption curve will be higher than the actual stability of the milk warrants. This error must be considered if we are to use the stability figures obtained in the manner described in the foregoing paragraphs to judge the stability of the milk samples when stored at lower temperatures. We do not feel that this possible cumulative error will influence in any way the conclusions of the paper.

With the exception of one more experiment, we have finished with the experimental work in this paper. This further point, which we will consider before proceeding to generalize upon the significance of the data, may be put as a question. Since normal forewarming (95°) unstabilizes condensed milk, and high forewarming (110–120°) stabilizes it, is the effect of the sugar the result of two separate reactions, one of which is brought about by high temperatures? This question cannot be answered definitely, but we are of the opinion that it is not. We reach this conclusion from the fact that a continued forewarming of the normal milk at 95° results in a stabilization of the product, although the effect

is not so marked as in the highly forewarmed product. The stability figures of milks forewarmed at 95° are given in Table V.

At the time of writing this paper portions of the milk samples described in this paragraph have been standing at room temperature for 60 days. The sample forewarmed for 20 minutes has solidified to a thick gel. The portion forewarmed for 30 minutes has thickened considerably, while the milk forewarmed for 45 minutes is still liquid, as are the other portions. These data emphasize the statements of the preceding paragraphs, where we

TABLE V.

*Stability Figures of Condensed Milks Forewarmed at 95°C. for Different Periods.*

Time of forewarming. min.	Stability figure.
0	242
10	226
20	230
30	250
45	232

call attention to the fact that there is a cumulative error in the value of our stability figures, which we explain as being caused by the removal of calcium from the field of action during the forewarming processes. But if we were to draw a curve showing the relative stability of these milk samples when stored at room temperature, this curve would be of the same type as that obtained when we plot our stability figures, containing a minimum and a maximum point. We are, therefore, justified, we believe, in reiterating our statement that this cumulative error in no way invalidates the conclusions of this paper.

## SUMMARY.

1. It is shown that an endothermic chemical reaction accompanies the coagulation of the casein of milk when the coagulation is brought about by heat.
2. This reaction is apparently the precipitation of calcium and magnesium as citrates and phosphates.

3. The metals combined with the protein are concerned in this reaction.

4. The thickening of condensed milk which sometimes occurs during the storage of the product is shown to be the result of a chemical reaction.

5. The thickening of the condensed milk can be prevented by a high forewarming (110–120°) and perhaps by a prolonged (30 minute) forewarming at 95°C.

6. The preliminary forewarming of evaporated milk for a 10 minute period, if done at 95°, stabilizes the finished product; but such forewarming, if done at 110° slightly unstabilizes, and if done at 120° decidedly unstabilizes, the finished product.

7. The preliminary forewarming of condensed milk for 10 minutes, if done at 95° unstabilizes the finished product; but if done at 110° it slightly stabilizes, and if done at 120° it decidedly stabilizes the finished product.

8. A prolonged forewarming of condensed milk at 95° is shown by heat absorption tests, first to unstabilize the product and then to stabilize it.

9. Sugar is shown to exert a very definite effect upon the stability of condensed milk. There is not enough data as yet to warrant conclusions as to the nature of the reaction.

10. A number of facts are cited which must be taken into consideration if a theory of coagulation is to be formulated. The authors do not feel justified in detailing any coagulation theory until more work upon the salt equilibrium in milk is completed. They do feel justified, however, in hazarding the opinion that the controlling factor which determines the stability of milks toward the action of heat is the equilibrium of the salts of the milk, and that the effects of forewarming upon the stability of milk samples under further heating are the effects produced by altering this equilibrium. Since sucrose solutions dissolve calcium phosphate and citrate, and since sucrose can also form compounds with both calcium and phosphorus, it seems probable that the effect of cane-sugar in condensed milk samples, as regards the stability of the samples, will also be explained by the action of the sugar upon that equilibrium. Lindet (7), as we have already stated, advances the theory that the coagulation reaction is the transfer of calcium from the protein to the triphosphate by the

acid di-basic phosphate. There are no data in this paper that contradict this theory. It may be of interest here to state that an artificial milk serum increases in acidity upon precipitation, while there is no material change in the acidity of thickened condensed milk samples. The pH of an artificial serum changes from 6.59 to approximately 6.25 upon precipitation. We believe that any denaturation or dehydration of the protein before precipitation by heat is a secondary reaction.

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## THE PREPARATION OF TRYPTOPHANE FROM THE PRODUCTS OF HYDROLYSIS OF LACTALBUMIN WITH BARYTA.

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Baryta as a hydrolyst for the indirect determination of tryptophane has been used by Homer (1) and by Onslow (2). The writer has attempted Hopkins and Cole's (3) direct isolation procedure with baryta hydrolysates. The indication of these experiments is that the baryta hydrolysis cannot be carried to completion without destruction of some of the tryptophane. For the preparation of tryptophane from proteins by Hopkins and Cole's method, however, this hydrolysis has been found to have some advantages over the trypsin digestion. It is much quicker; and it destroys cystine, in the absence of which the purification of the tryptophane is considerably simplified.

The following technique, in which lactalbumin is hydrolyzed with barium hydroxide and Hopkins and Cole's method for isolation of tryptophane is applied to the products, has been found satisfactory in convenience and yield.

The lactalbumin used was prepared by coagulation in the presence of a very small proportion of hydrochloric acid from the filtrate remaining after the preparation of casein by Clark's method.

200 gm. of the lactalbumin were treated with a solution of 700 gm. of recrystallized baryta in 4 liters of distilled water and heated on the steam bath for 40 hours at a temperature of about 85°. A small amount of organic substance remained undissolved to the end of the hydrolysis period. This gave no color with the Hopkins-Cole reagent, and was therefore discarded. Baryta was removed from the filtrate, with sulfuric acid, and a further

quantity of sulfuric acid added, so that the solution contained 7 per cent of sulfuric acid. The entire volume of the filtrate and washings of the barium sulfate was about  $8\frac{1}{2}$  liters at this point, and about 1 liter of the mercuric sulfate reagent of Hopkins and Cole (3) was required for the precipitation of tryptophane. The reaction mixture was allowed to stand for 3 days. The precipitate was then filtered off by suction in a cup filter of hardened paper in a small Buchner funnel and washed many times with 5 per cent sulfuric acid. The precipitate was thoroughly stirred up with the dilute acid, care being taken to crush all lumps. The precipitate was drained thoroughly after each washing. This process was continued until tyrosine could not be detected in the filtrate, from fifteen to twenty treatments carried out as described being found necessary. The precipitate was then placed in 5 per cent sulfuric acid, ground to a fine suspension, and the mixture saturated with hydrogen sulfide. The solid residue was filtered off, ground in a mortar with a fresh quantity of 5 per cent sulfuric acid, and again treated with hydrogen sulfide. This was continued until the filtrate from the mercuric sulfide no longer gave the characteristic color reaction of tryptophane. The resulting solution of tryptophane in sulfuric acid showed a tendency to darken rapidly. Alcohol was added in small quantities to prevent the decomposition thus indicated.

The sulfuric acid was removed from the solution quantitatively with baryta, the resulting barium sulfate was washed with hot distilled water containing a little alcohol until no more tryptophane was removed, and the filtrate, with the washings, was concentrated under diminished pressure, with frequent addition of small quantities of alcohol, until crystallization began. Decolorizing was not found necessary, almost all the color having been taken up by the barium sulfate. The concentration was then carried somewhat further at ordinary pressure. It was found that a better crystallization was secured in this way than by completing the concentration under diminished pressure. After removing all the tryptophane which could be crystallized, the mother liquor, with the filtrates from the recrystallizations of the crude products, was subjected to the further action of baryta, in 35 per cent solution, at the temperature of a steam bath for 48 hours. The baryta was quantitatively removed and a further

small quantity of crude tryptophane was crystallized. The filtrate from this crop yielded no crystalline substance on further concentration. This was treated as before with baryta, heating for about 60 hours, and a further small crop of crystals was obtained. Further treatment of the non-crystallizable residue was without result; though color reaction was still strong. The total yield of purified product was 2.64 gm., which was found on analysis to contain 13.47 per cent of nitrogen. (The theoretical nitrogen content of tryptophane is 13.75 per cent.) Under the microscope it showed the characteristic crystalline habit of tryptophane.

The yield on the basis of ash- and moisture-free lactalbumin used was 1.45 per cent.

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## PROTEINS OF THE CANTALOUP SEED, *CUCUMIS MELO*.

### ISOLATION OF A CRYSTALLINE GLOBULIN, AND A COMPARATIVE STUDY OF THIS GLOBULIN WITH THE CRYSTALLINE GLOBULIN OF THE SQUASH SEED, *CUCURBITA MAXIMA*.

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The extremely complex character of the protein molecule renders it difficult if not impossible by any known means at present available to determine the chemical individuality of any single protein, or to establish beyond question the identity of any two proteins from different sources. This complexity confers upon proteins such chemical and physical properties that the tests and methods commonly used to determine the identity of more simple compounds either cannot be applied or, when applied, give results which do not furnish conclusive evidence to this end. Two proteins may have the same crystal form, elementary composition, percentages of the same amino-acids, molecular weight, crystallographic and optical properties, even the same immunological reactions, and yet be distinctly different. All of these points of similarity together would not constitute indisputable proof that the proteins were identical, although they would furnish very strong evidence to that effect.

In this paper is described an investigation of the proteins of the cantaloupe seed, *Cucumis melo*. Two proteins were isolated from this seed—a globulin and a glutelin. A globulin preparation was obtained which consisted entirely of well formed octahedra. The crystal form, method of preparation, and elementary composition of this globulin so closely resembled those of the squash seed globulin as isolated and described by Osborne (1) that a comparative study of the two globulins from these seeds was

suggested, with the view of securing as much evidence as possible toward establishing either their identity or non-identity. For this purpose a crystalline globulin preparation from hulled squash seeds was prepared according to the method of Osborne (1). With but few exceptions the vegetable proteins which have been isolated in crystalline form have been globulins obtained from oil seeds, such as hemp seed, castor bean, Brazil nut, sesame seed, flaxseed, and squash seed (1). It is a rather remarkable fact that in practically all cases the crystalline globulins have been obtained as octahedra or as crystals belonging to the isometric system. The

TABLE I.

*Average Results of Duplicate Analyses of Squash Seed Globulin.\* A Comparison of the Elementary Composition of the Cantaloupe and Squash Seed Globulins.*

	Crystallized squash seed globulin.		Crystallized cantaloupe seed globulin (Preparation XIV).	Average analysis of fourteen preparations of cantaloupe seed globulin.		
	Preparation.					
	I	II				
	per cent	per cent	per cent	per cent		
C.....	52.19	52.14	52.17	52.74		
H.....	6.54	6.57	6.56	6.72		
N.....	18.39	18.63	18.51	18.42		
S.....	1.01	0.99	1.00	1.11		
O.....	21.87	21.67	21.76	21.01		
Moisture.....	6.99	9.51				
Ash.....	0.56	0.52				

\* Calculated on an ash- and moisture-free basis.

similarity of the globulins from the seeds of the squash and cantaloupe was first revealed by their practically identical behavior and physical properties exhibited during the process of their preparation.

Furthermore, from all of the chemical and physical data obtained in our study of the isolated and purified globulins of these two seeds, we have been unable to find any point of difference which would indicate their non-identity.

Table I gives figures showing the elementary composition of the two crystalline globulins. These figures represent the average

results of a large number of duplicate analyses. The small differences noted in their elementary composition are not greater

TABLE II.

*Distribution of Nitrogen in the Cantaloupe Seed Globulin as Determined by the Van Slyke Method.\**

Sample I, ash- and moisture-free, 2.6901 gm. protein, 0.4955 gm. nitrogen.†  
 " II, " " " 2.6901 " " 0.4955 " " †

	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N.....	0.0376	0.0371	7.59	7.49	7.54
Humin N adsorbed by lime.....	0.0035	0.0034	0.71	0.69	0.70
Humin N in ether-amyl alcohol extract.....	0.0004	0.0004	0.08	0.08	0.08
Cystine N.....	0.0039	0.0038	0.79	0.77	0.78
Arginine N.....	0.1411	0.1404	28.48	28.33	28.41
Histidine N.....	0.0303	0.0312	6.11	6.30	6.20
Lysine N.....	0.0166	0.0172	3.35	3.47	3.41
Amino N of filtrate.....	0.2500	0.2500	50.45	50.45	50.45
Non-amino N of filtrate.....	0.0106	0.0106	2.14	2.14	2.14
Total N regained.....	0.4940	0.4941	99.70	99.72	99.71

\* Nitrogen figures corrected for the solubilities of the phosphotungstates of the bases.

† Nitrogen content of protein, 18.42 per cent.

TABLE III.  
*Basic Amino-Acids of the Proteins.*

Amino-acids.	Cantaloupe seed globulin.	Squash seed globulin.	Cantaloupe seed glutelin.
	per cent	per cent	per cent
Cystine.....	1.23	1.26	1.07
" *.....	1.31	1.42	1.11
Arginine.....	16.26	15.69	12.42
Histidine.....	4.22	4.54	2.72
Lysine.....	3.29	3.70	4.59
Tryptophane†.....	2.63	2.92	3.03

\* By the colorimetric method of Folin and Looney (2).

† By the colorimetric method of May and Rose (3).

than those frequently met in the analysis of different preparations of the same protein.

A study of the distribution of the nitrogen (Tables II, III, IV, and V) in the two globulins as determined by the Van Slyke

TABLE IV.

*Distribution of Nitrogen in the Squash Seed Globulin as Determined by the Van Slyke Method.\**

Sample I, ash- and moisture-free, 2.7735 gm. protein, 0.5100 gm. nitrogen. †  
 " II, " " " 2.7735 " " 0.5100 " " †

	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N .....	0.0392	0.0394	7.69	7.73	7.71
Humin N adsorbed by lime.....	0.0053	0.0058	1.04	1.14	1.09
Humin N in ether-amyl alcohol extract.....	0.0004	0.0001	0.08	0.02	0.05
Cystine N .....	0.0040	0.0041	0.78	0.80	0.79
Arginine N.....	0.1397	0.1404	27.39	27.53	27.46
Histidine N.....	0.0342	0.0340	6.71	6.67	6.69
Lysine N.....	0.0196	0.0197	3.84	3.86	3.85
Amino N of filtrate.....	0.2602	0.2602	51.02	51.02	51.02
Non-amino N of filtrate.....	0.0033	0.0077	1.62	1.50	1.56
Total N regained.....	0.5109	0.5114	100.17	100.27	100.22

\* Nitrogen figures corrected for the solubilities of the phosphotungstates of the bases.

† Nitrogen content of protein, 18.39 per cent.

TABLE V.

*Distribution of Nitrogen in the Proteins as Calculated from the Van Slyke Analyses in Terms of Percentage of the Proteins.*

Nitrogen.	Cantaloupe seed globulin.*	Squash seed globulin.†	Cantaloupe seed glutelin.‡
	per cent	per cent	per cent
Amide.....	1.39	1.42	1.23 *
Humin.....	0.14	0.21	0.41
Basic.....	7.15	7.14	5.74
Non-basic.....	9.69	9.67	9.01
Total.....	18.37	18.44	16.39

\* Nitrogen content, 18.42 per cent.

† Nitrogen content, 18.39 per cent.

‡ Nitrogen content, 16.37 per cent.

method also gave results which agree within the limits of error of this method for duplicate analyses of the same protein.

In addition to the values found for cystine by the Van Slyke method, the content of this amino-acid was also determined colorimetrically by the recently published method of Folin and Looney (2). It is of interest to note the close agreement between the results obtained by the two different methods (Table III). Tryptophane was determined colorimetrically according to the method of May and Rose (3). The percentage of tryptophane found in the two globulins, together with those of the diamino-acids, is given for comparison in Table III.

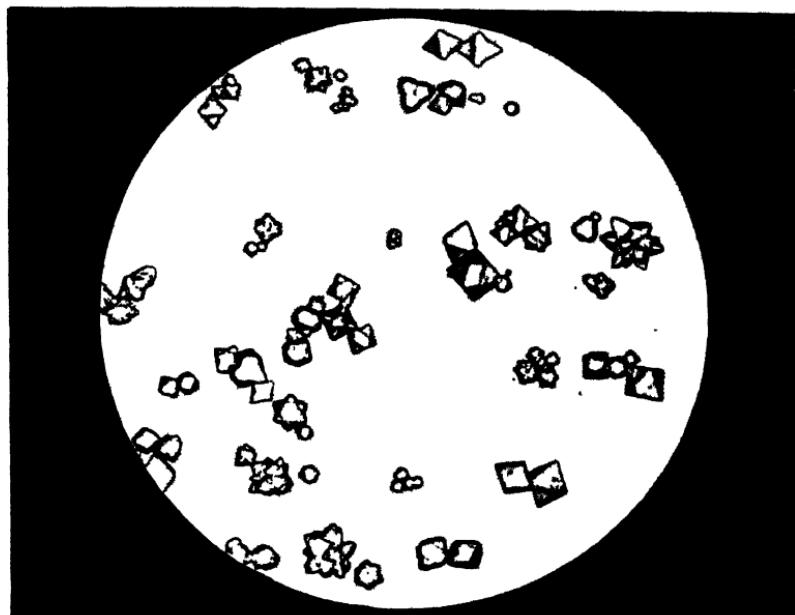


FIG. 1. Globulin from the cantaloupe seed, *Cucumis melo*.  $\times 210$ .  
Photomicrographs by G. L. Keenan.

The globulins of both the squash seed and cantaloupe seed were obtained in the form of regular, microscopic octahedra. Samples of the globulins were submitted to Dr. E. T. Wherry and Mr. G. L. Keenan of the Bureau of Chemistry, who kindly made the crystallographic and optical examinations and prepared the photomicrographs (Figs. 1 and 2). No difference in the crystallographic or optical properties of the two globulins was detected.

In view of the practically identical results obtained by the study of the chemical and physical properties of these two globulins it was of interest to compare them with respect to their immunological relations. Through the kind cooperation of Prof. H. G. Wells of the University of Chicago, we have been enabled to secure data to make this comparison. Guinea pigs sensitized with squash seed globulin reacted anaphylactically as severely to cantaloupe seed globulin as did those sensitized with the latter,

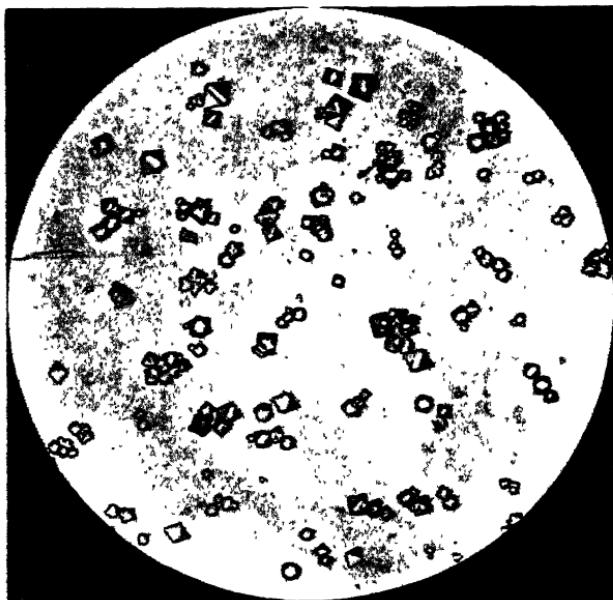


FIG. 2. Globulin from the squash seed, *Cucurbita maxima*.  $\times 210$ .  
Photomicrographs by G. L. Keenan.

and *nice versa*. No distinction immunologically could be observed between the globulins of the two seeds. The results of these tests are given in Table VI. These results are of special significance in the comparative study of these globulins, since Wells and Osborne (4) have pointed out that it seems probable that "the specificity of the anaphylaxis reaction is determined by the chemical structure of the reacting proteins rather than by their biological origin."

TABLE VI.  
*Anaphylaxis Experiments Made with the Crystallized Globulins of the Seeds of the Cantaloupe and Squash.\**

Experiment No.	Sensitizing dose (1 mg.).	Intoxicating dose (50 mg.).	Result.
1	Squash seed globulin. " " "	Squash seed globulin. " " "	Died, 2 hrs.
2	" " "	" "	" 1.75 "
3	" " "	Cantaloupe seed globulin. " " "	" 70 min.
4	" " "	" " "	" 2 hrs.
5	" " "	" " "	Moderately severe.
6	Cantaloupe seed globulin. " " "	" " "	" "
7	" " "	" " "	" "
8	" " "	Squash seed globulin. " " "	Died, 25 min.
9	" " "	" " "	" 40 "
10	" " "	" " "	Moderately severe.

\* These experiments were made by Prof. H. Gideon Wells, University of Chicago.

The collective data obtained in this investigation offer very strong evidence that the crystalline globulins of the seeds of the squash and cantaloupe are identical. This is of interest in connection with the fact that these globulins were obtained from the seeds of plants which, although belonging to the same botanical family, Cucurbitaceæ, are not only specifically different, but which belong to distinct genera.<sup>1</sup>

The cantaloupe seed globulin used in the aforementioned comparative studies was obtained from a meal prepared from hulled cantaloupe seeds containing 58.16 per cent of protein ( $N \times 5.68$ ). All the protein preparations used in the other general studies were obtained from a press-cake remaining after expression of the oil. This press-cake<sup>2</sup> included the hulls of the seeds, and contained 4.08 per cent of nitrogen, which when multiplied by the conventional protein factor 6.25, is equivalent to 25.50 per cent of protein. When the factor 5.68<sup>3</sup> is used, however, the more accurate protein content of 23.17 per cent is obtained. Although the globulin prepared from the press-cake containing the hulls could be obtained largely in the form of crystals, it was found that when meal from the hulled seeds was used the globulin could be readily obtained wholly as octahedra.

No difference in the composition of the proteins, whether obtained from meal containing the hulls or from meal without hulls, was observed.

By extraction of the finely ground meal with 5 per cent sodium chloride solution, a maximum yield of 12.07 per cent of globulin was isolated, based on the weight of the meal used. The residue further yielded on extraction with 0.5 per cent aqueous sodium hydroxide 5.78 per cent of glutelin.

The cantaloupe seed globulin was prepared by five different methods, all of which yielded preparations which gave the same results on analysis.

<sup>1</sup> This information was furnished by Dr. W. E. Safford, Economic Botanist, Bureau of Plant Industry, United States Department of Agriculture.

<sup>2</sup> The cantaloupe seed press-cake was furnished by the Oil, Fat and Wax Laboratory, of the Bureau of Chemistry, United States Department of Agriculture.

<sup>3</sup> This factor is based on the relative quantities of globulin and glutelin found in the meal, and their respective nitrogen contents.

Careful examination failed to reveal the presence of an albumin or of more than one globulin in the seed.

#### EXPERIMENTAL.

##### *Proteins of the Cantaloupe Seed.*

*Preliminary Experiments.*—Small quantities of finely ground meal prepared from cantaloupe seed press-cake were thoroughly mixed with various solvents, in the ratio of 10 cc. of solvent to 1 gm. of meal, and the mixture was allowed to stand at room temperature, with occasional stirring, for 1 hour. Nitrogen determinations made on aliquot portions of the extracts<sup>4</sup> showed that the maximum quantity of protein was extracted by a 5 per cent sodium chloride solution (Table VII). Further experimentation showed that an extraction period of 15 minutes was as effective as that of 1 hour.

Exhaustive extractions were made by successively extracting the meal five or six times with sodium chloride solution. After the last extraction the residue was thoroughly washed with distilled water, and then extracted twice for  $\frac{1}{2}$  hour periods with 0.5 per cent aqueous sodium hydroxide. Nitrogen determinations were made on aliquot portions of each extract. The results of these experiments are given in Table VII.

Precipitation tests showed that practically all of the protein could be precipitated from the sodium chloride extract of the meal by making the extract 0.25 saturated with ammonium sulfate, precipitation beginning at 0.2 of saturation. Increasing the concentration of ammonium sulfate up to complete saturation caused the further separation of but a mere trace of material. The unusually narrow range of precipitability with ammonium sulfate indicates the presence of only one globulin in the cantaloupe seed.

Experiments were made to ascertain as nearly as possible the total content of globulin and glutelin in the meal. For this purpose 200 gm. of the meal were exhaustively extracted with 5 per cent aqueous sodium chloride, and the joint extracts slightly acidified with acetic acid and boiled. The coagulum obtained,

<sup>4</sup> Acknowledgment is due to Mr. S. Phillips of this laboratory for the Kjeldahl nitrogen determinations made in the course of this investigation.

## Proteins of the Cantaloupe Seed

TABLE VII.  
*Extraction Experiments.*

Solvent.†	Preliminary* extractions.		Maximum extractions.					
	Protein extracted. (N × 6.25)	Extraction No. (N × 6.41)‡	Solvent.†	Experiment I.§		Experiment II.¶		Protein extracted. (N × 6.25) (N × 6.41)‡
	per cent	per cent		(N × 6.25)	(N × 5.41)‡	(N × 6.25)	(N × 6.41)‡	per cent
Distilled water.....	2.02	1.75	1	5 per cent NaCl.....	.....	11.71	10.14	12.33
0.5 per cent NaCl.....	3.73	3.23	2	5 " "	.....	2.79	2.42	4.96
1.0 " "	5.53	4.79	3	5 " "	.....	1.60	1.38	0.47
1.5 " "	5.79	5.01	4	5 " "	.....	0.60	0.52	0.11
2.5 " "	7.85	6.80	5	5 " "	.....	0.58	0.51	
4.0 " "	8.42	7.29	6	5 " "	.....	0.47	0.41	
5.0 " "	15.97	13.88	7	0.5 " NaOH.....	.....	6.14	6.14	5.92
7.0 " "	15.71	13.60	8	0.5 " "	.....	0.26	0.26	
10.0 " "	15.84	13.71		Total.....	.....	24.15	21.78	23.79
								21.39

\* The extractions were carried out at room temperature, for a period of 1 hour each, with frequent stirring.

† Solvents were used in the proportion of 10 cc. per gram of meal.

‡ This factor is based on the known nitrogen content of the globulin. The factor for glutelin is 6.25.

§ The extractions were carried out at room temperature, for a period of  $\frac{1}{2}$  hour each, except for the last two which were for  $\frac{1}{4}$  hour each.

¶ The extractions were carried out at room temperature, for a period of 24 hours each, except for the last two which were for  $\frac{1}{2}$  hour each.

|| These extracts when subjected to tests for protein gave negative results.

after a thorough washing with distilled water and drying, weighed 25.48 gm. (corrected for ash and moisture). This is equivalent to 12.07 per cent of the meal, or 52.09 per cent of the protein content of the meal, as calculated from the nitrogen ( $N \times 5.68$ ). Since previous examination had shown that the meal contained no albumin, these figures may be considered as representing the globulin content of the meal. The residue remaining from the above sodium chloride extractions was thoroughly extracted at room temperature with 0.5 per cent aqueous sodium hydroxide, and the dissolved glutelin isolated in the same manner as described in the case of the globulin. The yield of glutelin obtained was 11.55 gm., which represents 5.78 per cent of the meal, or 24.94 per cent of the total calculated protein.

*Preparation of the Globulin.*—For each preparation of the globulin from 200 to 400 gm. of meal were extracted with 5 per cent salt solution. Owing to the presence of rather coarse particles of hulls in the meal, the extracts were readily obtained perfectly clear by one filtration by suction through mats of filter paper pulp.

Preparations of the globulin were obtained by seven different methods. In all cases the precipitated proteins were finally washed and dried in the usual way.

1. Preparations X, XI, and XII were obtained in 3.65 per cent yield by directly dialyzing the salt extract against chilled running water for from 10 to 15 days. Two of these preparations consisted in part of octahedral crystals.

2. Dilution of the extract with 10 volumes of distilled water gave Preparation VIII, in 3.5 per cent yield.

3. Slight acidification of the extract with acetic acid, immediately followed by dilution with 10 volumes of water, resulted in somewhat greater yields of protein (5.75 per cent) than did dilution with water without acidification (Preparations IV, V, VI, and VII).

4. Preparations I, II, and III were obtained by making the extract 0.4 saturated with ammonium sulfate. The precipitate was removed by filtration, redissolved by addition of water, and the solution filtered in order to remove small quantities of undissolved material. The globulin was then precipitated from the clear solution by dialysis. The yield was 4.72 per cent.

5. By boiling the extract after first acidifying with acetic acid, Preparation IX was obtained in 8.27 per cent yield.

6. Dialysis of the extract against dilute alcohol yielded Preparation XIII in 5 per cent yield. This preparation was almost wholly composed of very small crystals. The extract was first dialyzed for 2 days against 35 per cent alcohol, and the dialysis continued for 10 days against 60 per cent alcohol. Every 2 days the old alcohol was replaced by fresh alcohol. Treatment of the precipitated protein with 5 per cent sodium chloride solution at 32°C. showed that most of it had become denatured during the dialysis against alcohol. The insoluble portion was composed largely of octahedral crystals. No further examination was made of the small fraction which dissolved in the salt solution.

7. The fact that we were unable to obtain preparations of the globulin which consisted *entirely* of well formed crystals suggested that the extracts contained a trace of foreign material derived from the hulls present in the meal. Cantaloupe seeds of the Rocky Ford variety were secured. The hulls were entirely removed by hand, the meats ground and extracted with ether. The meats represented 60.4 per cent of the total weight of the seeds, and 31.13 per cent of the whole seed consisted of oil. Preparation XIV was obtained from 50 gm. of the meal thus prepared by extraction with 800 cc. of 2 per cent sodium chloride solution. The salt solution was first warmed to 60°C. and the meal added with stirring. The mixture was again warmed for a short time to bring the temperature back to 60°C., and then immediately and rapidly filtered by suction. The protein began to separate in the filter flask almost at once. The filtrate together with the precipitate was warmed to 50°C., at which temperature nearly all of the precipitate redissolved. The warm solution was again filtered rapidly. The separated globulin was again redissolved by warming the mixture to 50°C. The warm solution was placed in a warm water bath and allowed to cool slowly at room temperature. The globulin, which separated on cooling and standing over night, constituted 28.21 per cent of the meal used, and was wholly composed of regular microscopic octahedra.

The average results of analyses of the various globulin preparations are given in Table VIII.

*Preparation of the Glutelin.*—The residues remaining after exhaustively extracting the cantaloupe seed meal with sodium chloride in order to remove all traces of globulin were extracted

TABLE VIII.  
Average Results of Duplicate Analyses of the Cantaloupe Seed Globulin.\*

	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII†	XIII†	XIV†	Average.
	per cent per cent														
C.....	52.71	52.53	52.53	52.57	52.74	52.77	52.69	52.84	52.61	52.46	52.67	52.63	52.56	52.74	52.65
H.....	6.67	6.62	6.66	6.70	6.64	6.64	6.83	6.35	6.66	6.71	6.69	6.70	6.76	6.72	6.67
N.....	18.45	18.47	18.48	18.56	18.23	18.38	18.40	18.36	18.25	18.43	18.34	18.42	18.55	18.42	18.41
S.....	1.13	1.12	1.19	1.18	1.13	1.16	1.10	1.16	1.03	1.13	1.04	1.19	1.13	1.11	1.13
O.....	21.04	21.26	21.14	20.99	21.21	21.05	20.98	21.29	21.45	21.27	21.26	21.06	21.00	21.01	21.14
Moisture.....	7.95	7.19	7.67	8.41	6.47	7.80	5.81	6.85	5.24	8.04	7.48	6.49	6.71	10.30	
Ash.....	0.46	0.26	0.51	5.05	4.27	3.89	4.94	3.40	6.99	3.36	0.66	4.25	9.77	0.03	

\* Calculated on an ash- and moisture-free basis.

† Crystallized.

for about 1 hour with 0.5 per cent aqueous sodium hydroxide. The clear alkaline solutions obtained after filtering the extraction mixture through paper pulp by suction were slightly acidulated with acetic acid. The resulting precipitate was washed with distilled water, redissolved by 0.2 per cent sodium hydroxide solution, and reprecipitated by acetic acid. This process was repeated several times, and the glutelin finally dried in the usual way. The average yield was 5.1 per cent of the meal used. Analyses of these preparations are given in Table IX.

*Properties of the Globulin and Glutelin.*—One of the most striking properties of the cantaloupe seed globulin is the ease with which it can be obtained in the form of well defined crystals by the

TABLE IX.

Average Results of Duplicate Analyses of the Cantaloupe Seed Glutelin.\*

	Preparation.						Aver- age.
	I per cent	II per cent	III per cent	IV per cent	V per cent	VI per cent	
C.....	55.23	55.06	55.26	55.26	55.27	55.09	55.20
H.....	6.99	7.08	7.08	7.03	6.97	6.96	7.02
N.....	16.13	16.17	16.37	16.37	16.61	16.01	16.28
S.....	0.87	0.94	0.89	0.92	0.90	0.88	0.90
O.....	20.78	20.75	20.40	20.42	20.25	21.06	20.60
Moisture.....	7.28	6.93	6.92	7.75	6.84	5.67	
Ash.....	0.43	0.91	0.91	0.97	0.08	0.72	

\* Calculated on an ash- and moisture-free basis.

method described on page 90. The crystals consisted of well formed octahedra with  $N_d^{20}$  = approximately 1.545. Photomicrographs of these crystals are shown in Figs. 1 and 2.

The globulin has a narrow range of precipitability with ammonium sulfate, ranging in 5 per cent sodium chloride solution from 0.2 to 0.3 of saturation.

In aqueous sodium chloride, slightly acidified with acetic acid, the globulin started to flocculate at 73°C. and was completely coagulated at 75°C.

The glutelin was obtained in granular and powdery form, the color ranging from pale, creamy buff to deep buff. It is extremely soluble in dilute alkalies and mineral acids.

Both proteins gave strong positive tests for tyrosine with Millon's reagent.

*Analyses of the Proteins by the Van Slyke Method.*—Duplicate samples of 3 gm. each of the globulin and glutelin were hydrolyzed by boiling for about 30 hours with 100 cc. of 20 per cent hydrochloric acid. The phosphotungstates of the bases were decomposed with a mixture of ether and amyl alcohol in the usual way. The results of the analyses are given in Tables II, III, V, and X.

TABLE X.

*Distribution of Nitrogen in the Cantaloupe Seed Glutelin as Determined by the Van Slyke Method.\**

Sample I, ash- and moisture-free, 2.7651 gm. protein, 0.4527 gm. nitrogen.†  
 " II, " " " 2.7651 " " 0.4527 " " †

	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N.....	0.0339	0.0341	7.49	7.53	7.51
Humin N adsorbed by lime.....	0.0109	0.0112	2.41	2.47	2.44
Humin N in ether-amyl alcohol extract.....	0.0004	0.0003	0.09	0.07	0.08
Cystine N.....	0.0035	0.0034	0.77	0.75	0.76
Arginine N.....	0.1103	0.1106	24.37	24.43	24.40
Histidine N.....	0.0212	0.0195	4.68	4.31	4.50
Lysine N.....	0.0239	0.0247	5.28	5.46	5.37
Amino N of filtrate.....	0.2422	0.2432	53.51	53.73	53.62
Non-amino N of filtrate.....	0.0066	0.0062	1.45	1.37	1.41
Total N regained.....	0.4529	0.4532	100.05	100.12	100.09

\* Nitrogen figures corrected for the solubilities of the phosphotungstates of the bases.

† Nitrogen content of the protein, 16.37 per cent.

*Determination of Tryptophane.*—Determination of the tryptophane content of the globulin and glutelin was carried out for the most part according to the colorimetric method described by May and Rose (3). Samples, equivalent to 0.1 gm. of the ash- and moisture-free proteins, were incubated for 24 hours at 35°C. with 100 cc. of 20 per cent hydrochloric acid containing 1 cc. of a 5 per cent solution of *p*-dimethylaminobenzaldehyde in 10 per cent sulfuric acid. After standing for 40 hours at room temperature, the intensity of the color developed was matched in a Bock-

Benedict colorimeter against a standard solution prepared by similarly treating 0.1 gm. of zein and 10 cc. of a 1.5 per cent solution of pure tryptophane.

A standard solution containing only tryptophane developed with the aldehyde reagent a shade of color different from that obtained with the hydrolyzed proteins. Consequently, the colors could not be accurately matched. This difficulty was eliminated by the use of zein. Control experiments had shown that the zein used contained no trace of tryptophane detectable by this method.

It may be noted that May and Rose took the tryptophane content of casein as their standard, and calculated the tryptophane in the proteins they examined on the basis that casein contains 1.5 per cent of tryptophane.

The values obtained for the tryptophane content of the cantaloupe seed globulin and glutelin are given in Table III.

*Determination of Cystine.*—Cystine was determined in several preparations of the proteins of the cantaloupe seed according to the colorimetric method of Folin and Looney (2). Quantities of the protein equivalent to 1 gm. each of the ash- and moisture-free material were hydrolyzed by boiling for 12 hours with 25 cc. of 20 per cent sulfuric acid, and the cystine in the hydrolysates was determined by colorimetric comparisons with a standard containing 1 mg. of cystine in 100 cc. of solution. The values thus obtained are in close agreement with those found by the Van Slyke method (Table III).

#### *Globulin of the Squash Seed.*

For the purpose of comparing the properties and composition of the cantaloupe seed globulin with those of the crystalline globulin of the squash seed, several preparations of the latter were made. Seeds of Hubbard squash were hulled by hand, the meats ground, and the oil was removed by extraction with ether. The meal thus obtained contained 10.43 per cent of nitrogen. The crystallized globulin was prepared, with but few modifications, according to the method described by Osborne (1), and according to which the cantaloupe seed globulin was obtained. An average yield of 30 per cent of the meal used was obtained.

These preparations consisted wholly of well formed octahedra (Fig. 2) with  $N_d^{20}$  = approximately 1.545. Optical and crystal-

lographic examination revealed no difference between these crystals and those similarly obtained from the seed of the cantaloupe.

Elementary analyses (Table I), determination of the nitrogen distribution and diamino-acids by the Van Slyke method (Tables III, IV, and V), and colorimetric determinations of tryptophane and cystine (Table III) were made in the manner already described in the case of the cantaloupe seed globulin.

#### *Anaphylactogenic Properties of the Globulins from the Squash and Cantaloupe Seeds.*

The globulin preparations used for the anaphylaxis tests were wholly composed of octahedral crystals. By intraperitoneal injections guinea pigs were sensitized with 1 mg. doses of the proteins, and after an interval of 3 weeks a second, or intoxicating, dose of 50 mg. was administered. The results obtained (Table VI) show that these globulins are identical as far as their immunological relation is concerned. The cantaloupe seed globulin was a little less soluble than the globulin of the squash seed in very weak alkalies, which probably accounts for the fact that slightly less severe reactions were obtained when the cantaloupe seed globulin was used for intoxicating than when the squash seed globulin was used. This difference in solubility is probably due to the smaller size of the crystals of the squash seed globulin (Fig. 2).

#### SUMMARY.

Two proteins from cantaloupe seed, a globulin and a glutelin, have been isolated and analyzed. From ether-extracted meal of the hulled seeds, 28.21 per cent of globulin was obtained by extraction with 2 per cent aqueous sodium chloride at 60°C. On cooling, the globulin separated from the saline solution wholly as octahedral crystals. Elementary analyses showed the globulin to have the following percentage composition: C 52.65, H 6.67, N 18.41, S 1.13. Analyses by the Van Slyke method and colorimetric determination of cystine and tryptophane gave the following results for the diamino-acids: arginine, 16.26; histidine, 4.22; lysine, 3.29; cystine, 1.27; tryptophane, 2.63 per cent.

A comparison of the chemical and physical properties of the cantaloupe seed globulin with those of the globulin of the squash

seed similarly prepared showed no point of difference between these two proteins. Anaphylaxis experiments on guinea pigs also showed that these two proteins could not be distinguished immunologically.

The cantaloupe seed glutelin was obtained in 5.78 per cent yield from an ether-extracted meal which contained the hulls (yield of globulin from the same meal was 12.07 per cent), by extraction with 0.5 per cent sodium hydroxide. Analyses of the glutelin gave the following results: C 55.20, H 7.02, N 16.28, S 0.90, arginine 12.42, histidine 2.72, lysine 4.59, cystine 1.09, tryptophane 3.03 per cent.

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## ON THE PHOSPHORUS COMPOUNDS IN NORMAL BLOOD.

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The study of the occurrence and distribution of phosphorus compounds in blood has been stimulated in recent years by the introduction of new analytical methods. These studies have led rather definitely to the opinion that there are present in human blood three distinct types of phosphorus compounds: (1) inorganic phosphates; (2) lipoids that contain phosphorus; and (3) organic substances other than lipoids that contain phosphorus. Classes (1) and (3) are referred to collectively as "acid-soluble phosphates." Class (3) is frequently spoken of as "organic phosphate" or "unknown phosphate." When substances of this latter class are boiled with mineral acid the phosphate group is split from the organic compound and can then be precipitated together with the inorganic phosphate by any reagent suitable for this purpose.

Nothing definite is known concerning the chemical identity of these organic phosphates. They have been shown to be unstable and soluble in dilute acids, but they are not soluble in the alcohol-ether mixture which dissolves lipoids. Zucker and Gutman<sup>1</sup> have recently shown that part of this unknown organic phosphate in human blood, and in rat's blood as well, is easily capable of hydrolysis, whereas another part cannot be hydrolyzed easily under the same conditions.

Forms of phosphoric acid present in blood, other than the three classes named, have been described occasionally.

While there are reported results and expressed opinions which indefinitely indicate the contrary, it has nevertheless been almost

<sup>1</sup> Zucker, T. F., and Gutman, M. B. *Proc. Soc. Exp. Biol. and Med.*, 1922-23, xx, 133.

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universally assumed that all of the three types of phosphorus compounds named above are present in both plasma and corpuscles. In fact it has even been stated that in regard to inorganic phosphates there is no distinction at all to be drawn between corpuscles and plasma. For instance, Zucker and Gutman<sup>2</sup> found that the amount of inorganic phosphate is the same in equal volumes of plasma and of whole blood, thus indicating that the inorganic phosphates of blood exist in the same concentration in plasma and corpuscles.

In some experiments undertaken in this laboratory for a different purpose such divergent results were obtained as to arouse the suspicion that this concensus of opinion is not correct. In fact it was found that in dog's blood certainly, and in human blood probably, the inorganic phosphates are confined to the plasma and the organic phosphates to the corpuscles.

### EXPERIMENTAL.

In the following determinations of phosphoric acid the general methods of Bloor<sup>3,4</sup> were used throughout. These methods were chosen in preference to others because they are adapted to the study of the problem in hand in so far as they are rapid, and do not involve any reagents that might be expected to produce decomposition of the material with which they are used. These considerations are particularly important when the labile character of the organic fraction is recalled. The strychnine molybdate reagent, by means of which the phosphate ion is precipitated as strychnine phosphomolybdate, distinguishes sharply between inorganic and organic phosphates. The precipitates so obtained are compared nephelometrically with precipitates obtained under standard conditions.

It is true that the greatest care must be used to prevent the introduction of even minute quantities of extraneous material and to control the conditions exactly, on account of the great sensitiveness of this reaction; but when properly controlled the sensitiveness of the reaction is its greatest asset.

<sup>2</sup> Zucker, T. F., and Gutman, M. B., *Proc. Soc. Exp. Biol. and Med.*, 1921-22, xix, 169.

<sup>3</sup> Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 33.

<sup>4</sup> Bloor, W. R., *J. Biol. Chem.*, 1920-21, xlv, 171.

*Dog's Blood.**1. Phosphates of the Corpuscles.**The Absence of Inorganic Phosphates from Corpuscles.*

The danger recognized by Bloor and others that the organic fraction may be decomposed with the liberation of inorganic phosphate during the manipulation incident to a determination, is a real one. Several control experiments were performed, in which the effect was studied of allowing solutions of corpuscles to stand for different lengths of time and at different temperatures at various stages of the determination. The results of these experiments proved that the decomposition of the organic fraction was appreciable at room temperature if the corpuscle solutions were allowed to stand before the proteins were precipitated by ammonium sulfate; but that the decomposition was slow even at 37° after the precipitation of the proteins. There is reason to believe that, if the determination is carried through rapidly, the whole process being done in the cold, the decomposition can be minimized or even prevented altogether.

In many of the experiments the blood was centrifuged in a tube surrounded by a bath of cracked ice, but this procedure was subsequently abandoned, for it was found that the advantage gained did not compensate for the additional time consumed. If the tubes were chilled at the beginning of the centrifugation the temperature did not rise high enough to effect an appreciable decomposition of the organic fraction.

*Stability of the Organic Fraction at Low Temperatures.*—Blood was drawn from the external jugular vein of a normal male dog directly into a centrifuge tube containing the minimum amount of oxalate necessary to prevent clotting. The tube was thrust into cracked ice immediately, and all the subsequent procedures were performed in the cold. After centrifugation in a graduated centrifuge tube an equal volume of 0.9 per cent sodium chloride was added. This chilled mixture was centrifuged again, and the washing process was repeated. The corpuscles were then hemolyzed with an equal volume of very dilute saponin solution, for 10 minutes, on ice, after which the proteins were precipitated with saturated ammonium sulfate made acid with acetic acid, in the

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usual manner. 2 cc. portions of the filtrate were added to 25 cc. of strychnine molybdate reagent. So slight a cloud was produced that it could not be compared nephelometrically with the weakest standard at hand; *i.e.*, one corresponding to 5 mg. of  $H_3PO_4$  per 100 cc. of corpuscles.

*Admissibility of Saponin as a Hemolytic Agent.*—The effect of saponin, in the quantities used, on the precipitation of phosphates by means of the strychnine molybdate reagent was determined repeatedly in various ways. Although saponin was found to exert an inhibitory effect upon the precipitation of pure phosphate solutions and also of phosphates in plasma, it had no demonstrable influence on the precipitation of the phosphates of whole blood or of corpuscles. Presumably the saponin was dragged down in the voluminous precipitation of proteins by acidified ammonium sulfate. In any event, the action of saponin can have no bearing on the ultimate question of the existence of inorganic phosphates in normal corpuscles, for the use of saponin was eventually abandoned.

The fact that there was only a trace of inorganic phosphate in the protein-free filtrate of the corpuscle solution suggested the possibility that this trace might be due to a slight imperfection in the execution of the method. In subsequent experiments, therefore, the procedure was varied in such a way as to test this possibility. For example, the corpuscles were washed four times with 0.9 per cent sodium chloride, and the tubes were packed in ice during centrifugation. In these experiments a small amount of inorganic phosphate was found (3.0 to 3.5 mg. of  $H_3PO_4$  per 100 cc. of corpuscles).

*The Part Played by the Anticoagulant.*—On the supposition that the oxalate which was used to prevent coagulation might in some way have produced diffusion an attempt was made to separate plasma from cells without the use of an anticoagulant.

Blood was drawn directly from the external jugular vein of a normal dog into a small centrifuge tube without the use of an anticoagulant. The blood was centrifuged immediately, speed alone being relied upon to prevent clotting. The only evidence of clotting was in the plasma in the zone extending for a distance of about 1 cm. immediately above the cells. There was no evidence of hemolysis. The corpuscles were washed four times with

0.9 per cent sodium chloride, and were hemolyzed for 10 minutes with an equal volume of very dilute saponin solution. The remainder of the procedure was identical with that of the previous experiment. There was not the faintest trace of precipitate or cloudiness visible when the final solution was added to the strychnine molybdate reagent. The solution remained as clear as distilled water, and was indistinguishable from the control standard in which all the reagents were added in the same quantities, with the omission of the standard phosphate solution. A special standard, corresponding to 3.0 mg. of  $H_3PO_4$  per 100 cc. of corpuscles, was prepared. When this standard was added to the strychnine molybdate reagent, the solution became unmistakably cloudy.

This experiment shows rather definitely that the trace of  $H_3PO_4$  under consideration, although negligible, is in fact to be attributed to the procedure and not to the existence of preformed  $H_3PO_4$  in the corpuscles.

Blood was collected and centrifuged as in the previous experiment. In this experiment the corpuscles were hemolyzed with an equal volume of distilled water, without the use of saponin. The whole process was performed in the cold. The complete absence of inorganic phosphates was demonstrated repeatedly in this manner. The final corpuscle solution, ready for precipitation of the phosphate, was allowed to stand for an hour surrounded by cracked ice and was found free from inorganic phosphates at the end of that time. A duplicate portion was allowed to stand for an hour at room temperature. A questionable trace of phosphate was found in this solution.

*On the Remote Possibility That Inorganic Phosphates Are Removed from the Corpuscles by Washing.*—In anticipation of the criticism that the inorganic phosphate might have been washed out of the corpuscles in the manipulation, several experiments were undertaken of which the following is typical.

Blood was drawn from a normal dog without the use of an anticoagulant. The blood was chilled and defibrinated. With careful manipulation hemolysis was avoided. Determinations were made of the inorganic phosphate of the whole (defibrinated) blood and of the serum obtained by centrifugation. Hematocrit readings were also made. On the basis of these data the theoretical value of the inorganic phosphate of the serum was

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calculated, on the assumption that the corpuscles contained no phosphate. The satisfactory agreement between the theoretical and determined values speaks for the validity of this assumption. Typical results are given in Table I.

### 2. Phosphate of Plasma.

*Effect of Oxalate on the Determination of Inorganic Phosphate of Plasma.*—In the light of the results of the previous experiments, a study of the influence of the oxalate on the determination of the inorganic phosphate of plasma became desirable.

A normal dog was bled, the blood allowed to clot, and the serum was obtained by centrifugation. The inorganic phosphate of this serum was found to be 19.2 mg. of  $H_3PO_4$  per 100 cc. of serum. To one portion of serum a liberal amount of oxalate was added,

TABLE I.  
*Inorganic Phosphate Content of Whole (Defibrinated) Dog's Blood.*

$H_3PO_4$ per 100 cc. of blood.	
Calculated.	Observed.
mg.	mg.
9.3	9.5
11.9	11.4
10.3	10.8

and to a second portion a large excess was added. The inorganic phosphates of these sera were found to be 18.5 and 19.2 mg., respectively. Apparently the addition of oxalate after the serum had been separated from the cells had no demonstrable effect on the inorganic phosphate.

Blood was drawn from a normal dog at one bleeding into four different tubes (about 10 cc. into each), containing, respectively: (1) no oxalate, (2) 20 mg. of oxalate, (3) 40 mg. of oxalate, and (4) 80 mg. of oxalate. The blood containing no oxalate was centrifuged immediately to prevent clotting. The other samples were allowed to stand on ice for 15 minutes before centrifugation. The plasmas were all analyzed for inorganic phosphates with the results given in Table II. There was marked hemolysis in (3) and (4).

The corpuscles from each sample were washed four times with 0.9 per cent sodium chloride and were then hemolyzed with distilled water (on ice for 10 minutes), and the inorganic phosphates were tested for as usual. (1) gave a water-clear solution. (2), (3), and (4) gave questionable traces of phosphate.

15 cc. of blood were drawn from a normal male dog, at one bleeding, into each of three flasks, containing (1) no oxalate, (2) 10 mg. of oxalate, and (3) 40 mg. of oxalate, respectively. The first was centrifuged immediately, as rapidly as possible. The second and third were thrust into cracked ice, and were allowed to remain 15 minutes before being centrifuged. The inorganic phosphate of these plasmas was determined, and found to be (1) 16.7 mg., (2) 15.2 mg., and (3) 13.9 mg. of  $H_3PO_4$  per 100 cc. of plasma,

TABLE II.

Determination No.	Oxalate added.	Inorganic phosphate obtained.
	mg.	mg.
I	0	16.6
II	20	15.5
III	40	15.0
IV	80	14.2

respectively. The corpuscles from (1) and (3) were washed four times with 0.9 per cent sodium chloride and inorganic phosphate in the hemolyzed corpuscle solutions eventually obtained was tested for. (1) gave no trace of inorganic phosphates, (3) gave a distinct trace.

*Effect of Sodium Citrate on the Determination of the Inorganic Phosphate of Plasma.*—Sodium citrate was found to be without effect on the determination of inorganic phosphates in plasma.

15 cc. of blood were drawn from a normal female dog into each of three flasks containing (1) no anticoagulant, (2) 60 mg. of sodium citrate, and (3) no anticoagulant. (1) was centrifuged immediately to prevent clotting. (2) and (3) were allowed to stand about 20 minutes on ice before centrifugation. Inorganic phosphates were found as follows: (1) 24.1 mg., (2) 24.1 mg., (3) 24.6 mg. of  $H_3PO_4$  per 100 cc. of plasma or serum.

These results were obtained consistently; *i.e.*, the inorganic phosphate of serum obtained under these conditions was identical with that of plasma obtained without the use of an anticoagulant.

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Occasionally an oxalated plasma gave identical values, but the use of a small excess of oxalate (in reality a "safe" excess) gave slightly low values for inorganic phosphate. Even though larger quantities of citrate than of oxalate were necessary to prevent coagulation, the citrated plasmas gave the same values for inorganic phosphate as did the plasmas obtained without the use of an anticoagulant.

*Organic Phosphate in Plasma.*—The results of the analysis of the plasmas in the previous experiments were particularly significant in view of the relation which had already been found between inorganic and acid-soluble phosphates in plasma. In a series of experiments the inorganic phosphate content of plasma had been found to be so nearly identical with the acid-soluble phosphate as to raise the question whether or not the difference between these two values (which determines the organic or unknown phosphate), might be due to experimental error. Occasionally, however, a large enough difference in these values was obtained to make this difference seem real. Consequently, the suggestion arose that these differences between inorganic and acid-soluble phosphates, not only in this work but also in the literature, might not be real differences, but merely secondary changes brought about by manipulation of the blood, notably by the action of oxalate or some other agent causing hemolysis.

Plasmas obtained from normal dogs as previously described without the use of an anticoagulant, were analyzed for inorganic and acid-soluble phosphates, the whole process of the determination of the inorganic phosphate being executed in the cold and carried through without any delay. Results given in Table III are typical.

Recently,<sup>5</sup> Bloor's ashing process has been criticized on the basis that significant amounts of phosphoric acid are volatilized. Although it is not clear whether or not this criticism was intended to apply also to the determination of acid-soluble phosphates, it was deemed wise to check this possibility. Solutions were prepared by mixing the ammonium sulfate reagent and inorganic phosphate solutions in appropriate amounts. These solutions were then treated as in an ordinary determination of acid-soluble phosphate with the exception that the distillation products were

<sup>5</sup> Baumann, E. J., *Proc. Soc. Exp. Biol. and Med.*, 1922-23, xx, 171.

collected in water. The distillates gave no reaction with strychnine molybdate reagent, even when considerable quantities of mixed nucleotides, prepared from yeast nucleic acid, were added to the original solutions before oxidation. Furthermore, the inorganic phosphate used in the oxidation could be recovered quantitatively, within the limits of accuracy of the determination. It is true that all the organic phosphate may not have been hydrolyzed under these conditions, but the fact that occasional high values were obtained when hemolyzed plasmas were analyzed demonstrates that, when present in significant amounts, this type of compound is hydrolyzable under these conditions.

TABLE III.

Determination No.	$H_3PO_4$ per 100 cc. of dog's plasma.	
	Inorganic phosphate.	Acid-soluble phosphate.
	mg.	mg.
I	15.2	15.6
II	16.8	16.7
III	16.4	16.8

The close agreement between the values for inorganic and acid-soluble phosphates in plasma obtained without the use of an anticoagulant, makes the existence of an organic phosphate in circulating plasma more than questionable.

#### *Human Blood.*

In view of the complete absence of inorganic phosphate in dog's corpuscles and of organic phosphate in dog's plasma it became a matter of interest to reinvestigate human blood in this respect.

Blood was drawn from a normal man by venepuncture, and was defibrinated on ice. Considerable hemolysis occurred. The inorganic phosphate of whole blood was found to be 6.0 mg. of  $H_3PO_4$  per 100 cc. of blood; of plasma 9.4 mg. of  $H_3PO_4$  per 100 cc. of plasma; of corpuscles 3.3 mg. of  $H_3PO_4$  per 100 cc. of corpuscles. On the basis of the first two determinations and of the hematocrit reading, the calculated inorganic content of the corpuscles was 2.6 mg. of  $H_3PO_4$  per 100 cc. of corpuscles. The acid-soluble phosphate value of the plasma was 10.7 mg. of  $H_3PO_4$  per 100 cc. As

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was invariably the case whenever hemolysis took place, a small amount of inorganic phosphate was found in the corpuscles, and of organic phosphate in the plasma.

This experiment was repeated twice with blood from different individuals, with closely similar results.

Blood was drawn by venepuncture from a normal man without the use of an anticoagulant, and was centrifuged rapidly. The corpuscles were washed four times with 0.9 per cent sodium chloride in the usual manner, and inorganic phosphates were tested for in the usual way. A trace of inorganic phosphate was found, too small to be determined nephelometrically, which corresponded approximately with a value of 1.5 mg. of  $H_3PO_4$  per 100 cc. of corpuscles.

Blood was drawn by venepuncture from a normal man and was prevented from clotting by the use of the minimum amount of oxalate. There was no hemolysis. The inorganic phosphate of the whole blood was found to be 6.8 mg. of  $H_3PO_4$  per 100 cc., of plasma 9.8 mg. of  $H_3PO_4$  per 100 cc., and of corpuscles 2.7 mg. per 100 cc. The calculated value for corpuscles was 3.0. The acid-soluble phosphates of the plasma amounted to 10.1 mg. per 100 cc.

### *Rabbit's Blood.*

Attempts were made to perform with rabbit's blood experiments similar to those which were made with dog's blood and human blood. Blood was obtained directly from the heart. Considerable difficulty was experienced in completely precipitating the protein from rabbit's blood and corpuscle solutions by means of the ammonium sulfate reagent. In two cases clear filtrates from corpuscle solutions were obtained, and only indeterminable traces of inorganic phosphate were found. The inorganic phosphate of the serum obtained from the defibrinated blood varied in amount from 12 to 17 mg. of  $H_3PO_4$  per 100 cc. In every case there was no demonstrable amount of organic phosphate present in these sera.

### DISCUSSION AND SUMMARY.

Evidence has been given which supports the conclusion that in the corpuscles of normal dog's blood there is no inorganic phosphate and in the plasma there is no organic phosphate. The

inorganic phosphate of 79 specimens of plasma from 10 dogs averaged 14.4 mg. of  $H_3PO_4$  per 100 cc. of plasma with extreme variations of 33.5 and 11.6 mg. The occasional traces of inorganic phosphate found in corpuscles and of organic phosphate in plasma apparently are due to postmortem changes. These bloods were usually drawn when the animals were in the postabsorptive state, but the same relation was found to obtain in the height of digestion.

Unusually low values were found for inorganic phosphates in human corpuscles (as low as 1.5 mg. of  $H_3PO_4$  per 100 cc. of corpuscles). Bloor obtained an average figure of 18.7 mg. of  $H_3PO_4$  per 100 cc. of corpuscles for men, and 15.7 mg. for women. Although solutions of human corpuscles were not obtained which were entirely free from inorganic phosphate (as was the case with dog's corpuscles), the small amounts found, taken together with the established ease of decomposition of the organic phosphate of the corpuscles, suggest that in the living organism there is no inorganic phosphate in human corpuscles, and that the small amounts found represent postmortem changes. This point of view is not in keeping with the statement that the concentration of inorganic phosphate of serum and of whole blood in fresh specimens is identical.



## A QUANTITATIVE METHOD FOR THE DETERMINATION OF PHENOLS IN BLOOD.

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Since the attempt of Benedict and Theis (1) in 1918 to apply the general procedure of Folin and Denis (2) to the determination of phenol in blood, there seems to have been no other effort in this direction except that of Pelkan (3). The latter has pointed out some of the shortcomings of the method of Benedict and Theis. Not only is their method cumbersome, involving as it does an estimation of uric acid, a determination of uric acid *plus* phenol, and a calculation of phenol by difference—thus magnifying the error—but in addition certain other features of the procedure make it impossible to obtain any likely index of conjugated phenols. The method proposed by Pelkan, on the other hand, although yielding more reliable results than the earlier one, would also seem to be open to some objections. The precipitation of proteins is sufficiently different from the widely used method of Folin and Wu (4) to make it impossible to use the same, now very familiar, tungstic acid filtrate which is used in so many other connections. Moreover, the removal of uric acid by silver lactate necessitates an extra filtration and often considerable trouble to eliminate excess silver. And lastly, the intensity of color obtained is hardly as great as might be desired.

While investigating the uric acid method of Morris and Macleod (5) the possibility suggested itself of utilizing for the determination of phenols the clear solution remaining after the precipitation of uric acid by zinc chloride and sodium carbonate. This proved feasible and was accordingly made the basis of the method herewith proposed. In this way it is possible to estimate uric acid and phenols, both free and conjugated, in an amount of filtrate easily obtained from 5 cc. of blood or plasma, or, in case duplicates are desired, from 8 to 10 cc.

### *Optimum Conditions for Color Development.*

A study was first made of the conditions producing the maximum intensity of color when the reagent of Folin and Denis is added to phenol solutions of as low a concentration as that in the blood filtrate. Accordingly, a large number of experiments were made

on very dilute phenol solutions to determine the optimum conditions of alkalinity, etc. The addition of sodium sulfite was dispensed with, but the intensifying effect of sodium cyanide was found to be sufficient to justify its use. This is in agreement, also, with the more recent methods for color development by uric acid, notably the results of Folin (6), Benedict (7), and Morris and Macleod. It was found inadvisable, however, to use sodium cyanide alone to render the solution alkaline, for the cyanide produces by itself a blue color with the reagent.

The fine white precipitate, which has apparently caused so much trouble in earlier work, it was found possible to avoid entirely if care was taken to avoid any great excess of the color reagent. The reagent used throughout this work was Bell's modification of the Folin-Denis reagent (8). (There would seem to be no reason to doubt that the reagent described by Wu (9) would yield results of exactly the same sort.) This reagent was diluted with 3 volumes of water, and 0.5 cc. of the diluted reagent was found sufficient to develop color with at least two or three times the amount of phenol normally present in 10 cc. of the filtrate.

The optimum conditions for color development were eventually fixed as follows:

To 10 cc. of solution containing about 0.030 mg. of phenol, 0.5 cc. of the diluted phenol reagent, 2 cc. of 20 per cent sodium carbonate, and 1 cc. of 5 per cent sodium cyanide are added. The sodium carbonate must be allowed to destroy the excess reagent before the cyanide is added, else the latter will produce a small but appreciable amount of color. Due to the dilution, however, this point has not the importance that Chapin (10) assigns to it in the case of sodium sulfite.

When allowed to stand at room temperature the color, developed under these conditions, increases in intensity for about an hour, at the end of which time it reaches a maximum and remains permanent almost indefinitely. To hasten the color development, however, the solution may be heated in boiling water for  $1\frac{1}{2}$  minutes. Heating too long results in a somewhat diminished color intensity.

Under these conditions of concentration blank determinations yield no color whatever. This will be dependent, however, upon the cyanide solution. Not only should this reagent be chemically pure, but the solution should preferably not be too fresh.

*Relations between Different Phenols.*

Benedict and Theis and Pelkan have used a standard solution of resorcinol throughout for comparison, with an arbitrary factor to convert results to a phenol basis. Resorcinol had been found to give 86 per cent of the color yielded by an equal weight of phenol. This empirical factor was redetermined by the present method, as were also the factors for *o*- and *p*-cresol.

Solutions of phenol, *o*-cresol, and *p*-cresol were prepared by weighing out about 5 gm. of the pure substance and dissolving in 1,000 cc. of water containing a few drops of concentrated HCl. These solutions were analyzed by the method of Messinger and Vortmann (11) and found to have the following concentrations.

	<i>mg. per cc.</i>
Phenol	5.53
<i>o</i> -Cresol	4.62
<i>p</i> -Cresol	5.33

These solutions were diluted 1:2,000, and their respective color-producing capacities compared by the present method with a standard solution of resorcinol prepared according to the directions of Benedict and Theis, containing 0.0232 mg. per cc. The ratio of resorcinol to the other three phenols as the result of a large number of determinations was as given in Table I.

The results from *o*-cresol were somewhat more variable than from the others and the color was a trifle off shade. Unfortunately, no pure *m*-cresol was available to include with the others.

The resorcinol standard of Benedict and Theis (0.581 mg.) instead of being equivalent to 0.5 mg. of phenol is, under the conditions of this method at least, equivalent to 0.637 mg. of phenol. The ratios of phenol to *o*- and *p*-cresol, 1.08 and 1.28, respectively, are in fairly close agreement with the similar ratios determined by Chapin who obtained 1.09 and 1.22 in these two cases.

In all subsequent work the solution of phenol was used as a standard. This solution was found to have undergone no deterioration whatever after standing a month. Although the weighing of pure phenol is uncertain, the determination of the concentration of pure solutions by the iodometric titration of Messinger and Vortmann is simple and rapid. Consequently, since the results

of "phenol" determinations are to be expressed in terms of *phenol*, the use of resorcinol standards and an arbitrary conversion factor would seem inadvisable for accurate work.

*Ratio between Color Intensity and Concentration of Phenol.*

In view of Benedict's admonition in his method for the colorimetric estimation of uric acid to keep the standard and unknown within a narrow range of difference it seemed advisable to ascertain the range of proportionality between concentration and color intensity in the present method.

TABLE I.

Resorcinol equivalent to 1 mg. of:		
Phenol.	<i>p</i> -Cresol.	<i>o</i> -Cresol.
mg.	mg.	mg.
0.910	0.725	0.772
0.915	0.703	0.795
0.936	0.710	0.772
0.925	0.703	0.765
0.883	0.724	0.745
0.896	0.714	0.794
0.910	0.704	0.720
0.925	0.704	0.781
0.913	0.714	0.809
0.896	0.711	0.772
0.925	0.725	0.779
0.910	0.718	
0.910		
0.896		
0.908		
0.910		
Average....0.911	0.713	0.77(3)

Accordingly, solutions of pure phenol of widely differing concentration were prepared by dilution of one standard, all subjected to the same procedure in the development of color, and all compared against the color of one standard solution of intermediate concentration. The theoretical concentration of this standard was 0.0267 mg. per 10 cc. The known amounts of phenol in 10 cc. of the other solutions and the amounts "found" by comparison against the standard were as shown in Table II.

Fig. 1 shows the results of plotting the amounts "found" against the amounts "present." The straight line is the ideal condition representing perfect proportionality. The crosses represent the actual results. It can be seen that the amount of phenol may safely be as little as half, or as much as twice, the

TABLE II.

Present. mg.	Found. mg.	Ratio. Found: present.	Present. mg.	Found mg.	Ratio. Found: present.
0.0053	0.0074	1.40	0.0267	(Standard.)	1.00
0.0085	0.0091	1.07	0.0320	0.0318	0.99
0.0107	0.0108	1.01	0.0373	0.0367	0.98
0.0128	0.0129	1.01	0.0426	0.0423	0.99
0.0160	0.0150	0.94	0.0533	0.0525	0.99
0.0213	0.0212	0.99	0.0800	0.0747	0.93

Each result is the average of at least three closely agreeing determinations.

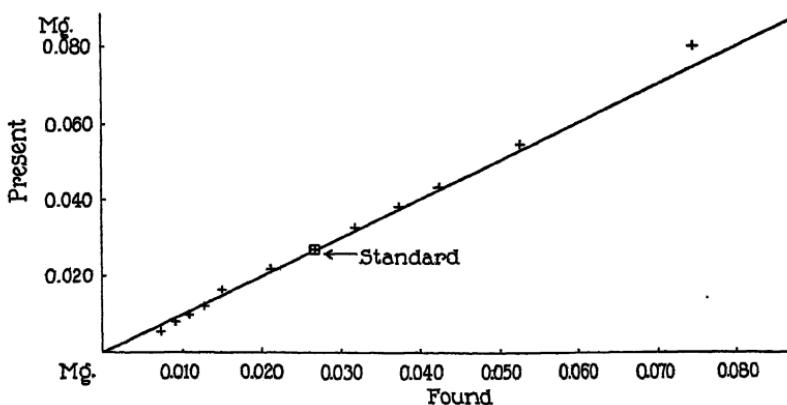


FIG. 1. Ratio between color intensity and concentration of phenol.

amount in the standard and still retain satisfactory proportionality between color intensity and concentration.

#### *Separation of Uric Acid and Phenol.*

Pelkan seems to have obtained unsatisfactory results in the separation of uric acid and phenol by precipitation with zinc

## 114 Determination of Phenols in Blood

chloride and sodium carbonate. The present writer has experienced no such difficulty. Not only was complete separation obtained, but no traces of zinc could be found in the filtrate.

To 25 cc. of solutions containing both uric acid and phenol were added 1 cc. of 2.5 per cent zinc chloride and, after mixing, 1 cc. of 10 per cent sodium carbonate. The precipitate was allowed to floccu-

TABLE III.

Uric acid.			Phenol.			Time between precipitation and centrifuging. hrs.
Present.	Found.	Recovered. per cent	Present.	Found.	Recovered. per cent	
<i>mg.</i>	<i>mg.</i>		<i>mg.</i>	<i>mg.</i>		
0.125	0.102		0.0667	0.0663		
	0.105			0.0667		
	0.103					
Average ....	0.103	83	Average ....	0.0665	100	0
0.125	0.114		0.0667	0.0661		
	0.114			0.0641		
	0.116			0.0658		
Average ....	0.115	92	Average ....	0.0653	98	2
0.125			0.0667	0.0631		
				0.0666		
			Average ....	0.0648	97	5½
0.125	0.119		0.0667	0.0630		
	0.122			0.0640		
Average ....	0.120	96	Average ....	0.0635	95	5
0.125	0.108	86	0.0667	0.0649	97	24
0.125			0.0667	0.0660	99	24
0.125			0.0667	0.0649	97	0 Precipitated with double amounts of $ZnCl_2$ and $Na_2CO_3$ .

late and settle somewhat and then was centrifuged. Phenol was determined in the supernatant liquid by subjecting 10 cc. to the procedure outlined above and uric acid was determined in the precipitate by the method of Morris and Macleod. Between 95 and 100 per cent of the phenol was recovered in all cases, as shown in Table III.

The time allowed for the precipitate to settle before removal has no effect upon the phenol recovery, but apparently has some influence on the recovery of uric acid.

Uric acid produces one-fourth to one-third of the color produced by phenol and small amounts of the former which may escape precipitation will have only a slight influence upon the recovery of phenol. The latter, on the other hand, produces no color whatever with the uric acid reagent of Morris and Macleod, or that of Benedict.

Proteins and certain amino-acids, notably tryptophane and tyrosine (as well as a few other substances), are also known to yield color with the phenol reagent. Tryptophane and tyrosine, in amounts which might reasonably be expected to exist in the blood, were added to phenol solutions and found to produce a small but not serious increase in the color intensity. Such interference seems unavoidable, however, and it must be kept in mind that "phenol," as determined by the present method, includes all non-protein constituents of the blood which are not precipitable by zinc chloride and sodium carbonate and which yield a blue color with the phenol reagent of Folin and Denis.

#### *Procedure of the Method.*

The reagents used were:

1. Sodium tungstate, 10 per cent.
2.  $\text{H}_2\text{SO}_4$ ,  $\frac{2}{3}$  normal.
3.  $\text{ZnCl}_2$ , 2.5 per cent.
4.  $\text{Na}_2\text{CO}_3$ , 10 per cent.
5.  $\text{Na}_2\text{CO}_3$ , 20 per cent.
6. Phenol reagent (Bell's modification). 100 gm. of sodium tungstate, 20 gm. of phosphomolybdic acid, 50 cc. of 85 per cent  $\text{H}_3\text{PO}_4$ , 100 cc. of concentrated HCl. Gently refluxed for 2 hours with 750 cc. of water, and then diluted to 1,000 cc. Dilute a portion of this with 3 volumes of water before using.
7.  $\text{NaCN}$ , 5 per cent. Add 3 to 4 drops of concentrated  $\text{NH}_4\text{OH}$  to 100 cc. of the solution. Folin recommends partial access of air to the solution, as in a beaker covered with a watch-glass; or stopper the bottle with a cotton plug.
8. Standard solution of phenol. Dissolve about 5 gm. of pure phenol in 1,000 cc. of  $\text{H}_2\text{O}$  and add 4 to 5 drops of concentrated

HCl. The exact concentration of the solution is then determined by the method of Messinger and Vortmann, as follows:

To 10 cc. add 6 cc. of approximately normal NaOH. Warm to 65°C. and add slowly a measured excess (40 to 50 cc.) of normal iodine solution. When the precipitation of iodophenol is complete, cool the solution, acidify with diluted H<sub>2</sub>SO<sub>4</sub>, and make up to 250 cc. Filter, and determine the excess iodine in an aliquot (100 cc.) of the filtrate by titration with standard thiosulfate solution. Calculate the iodine combined with the phenol. This multiplied by 0.1235 is the amount of phenol in 10 cc.

This stock solution will keep for several weeks at least. 10 cc. of this diluted to 500 cc. affords a solution which can be used for several days. The dilute standard used for determinations is made by dilution of 25 cc. of this second solution to 1,000 cc. 10 cc. of this will then contain about 0.025 mg. of phenol.

9. NaOH, 20 per cent. 20 gm. dissolved in 100 cc. of water.

Precipitate proteins by the method of Folin and Wu, laking the blood with 7 volumes of water and then adding 1 volume of 10 per cent sodium tungstate and 1 volume of  $\frac{2}{3}$  normal sulfuric acid. Let stand for some time and then filter. Following the directions of Morris and Macleod precipitate uric acid in 25 cc. of the filtrate by the addition of 1 cc. each of 2.5 per cent zinc chloride and 10 per cent sodium carbonate. Stir and let stand for an hour. Centrifuge to remove the precipitate, in which uric acid may be determined; or the solution may be filtered if there is no objection in recovering uric acid. To 10 cc. of the filtrate add 0.5 cc. of the diluted phenol reagent and 2 cc. of 20 per cent sodium carbonate. Mix and allow  $\frac{1}{2}$  minute for the destruction of the excess reagent. Then add 1 cc. of 5 per cent sodium cyanide. Immerse in boiling water for 1½ minutes. Cool in running water for at least 3 minutes and compare against a standard phenol solution, containing 0.025 to 0.030 mg. of phenol in 10 cc., which has been subjected to the same procedure as the unknown.

For determinations in plasma and corpuscles the directions of Wu (12) should be followed in the precipitation of proteins, using for plasma 8 volumes of water and  $\frac{1}{2}$  volume of each of the two precipitating reagents, and for corpuscles 5 volumes of water and 2 volumes of each of the reagents.

It has been observed that if clear-cut precipitations of the proteins and of the uric acid are not obtained, necessitating repeated

filtrations and additions of precipitating reagents, there seems to be a tendency to carry down some of the phenol in the precipitates.

*Recovery of Phenol Added to Blood.*

Phenol was determined by this method in samples of normal human and dog's blood and in samples of the same blood to which known amounts of phenol had been added, with the results given in Table IV.

*Total (Free Plus Conjugated) Phenols.*

Since no pure potassium phenylsulfate or other form of conjugated phenols was available it was impossible to investigate the completeness with which conjugated phenols are hydrolyzed by heating with acid. In view of the results of previous workers,

TABLE IV.

Origin.	Phenol found per 100 cc.	Difference.	Amount added.	Recovery.
Dog.	mg.	mg.	mg.	per cent
	2.35		None.	
	2.94	0.59	0.53	110
Human.	3.44	1.09	1.07	102
	2.88		None.	
	5.37	2.49	2.67	93
	1.86		None.	
	2.91	1.05	1.07	102
	3.16	1.30	1.33	97.5
	4.38	2.52	2.67	94

however, it seemed safe to assume that conjugated phenols are completely liberated by heating for 10 minutes at 100°C. in the presence of a few drops of concentrated HCl. Accordingly, attention was directed solely to establishing conditions under which pure phenol solutions might be subjected to such treatment and still maintain their color-producing property unchanged when compared with standards which had not been heated. This proved feasible if certain precautions were observed.

In the first place, the production of the blue color is very sensitive to changes in alkalinity, and solutions to be comparable,

must be kept within a fairly narrow range in this respect. HCl added both to standard and unknown must be partially neutralized before development of the color, but an excess of alkali must be avoided. During the heating it is advisable to close the tubes completely, to avoid not only possible loss of phenol by volatilization but also change in the volume of solution. A piece of large rubber tubing and a clamp-screw can be used for this purpose, but the rubber tubing must first be boiled in dilute acid and then in water, to remove traces of something on the inner surface which interferes by increasing the color intensity. The following details were eventually fixed upon.

To 10 cc. of filtrate is added 0.25 cc. of concentrated HCl, the tube closed with rubber tubing and a clamp-screw and heated in boiling water for 10 minutes. The same amount of HCl is also

TABLE V.

Origin.	Phenol per 100 cc.		
	Free. mg.	Total. mg.	Conjugated. mg.
Dog.	2.91	3.10	0.19
	2.35	2.76	0.41
Human.	1.86	2.34	0.48
	1.80	1.97	0.17
	2.43	3.23	0.80
	1.84	2.06	0.22

added to 10 cc. of the standard solution, which is not heated. The solution is cooled and both to unknown and standard is added 0.5 cc. of 20 per cent NaOH. This nearly, but not quite, neutralizes the HCl. Mix, and proceed as in the determination of free phenol. The difference between total and free represents conjugated phenol.

Pure solutions of phenol, known to contain 0.0267 mg. in 10 cc. subjected to this process, yielded 0.0267, 0.0265, 0.0272, and 0.0276 mg., respectively, showing that the procedure has no effect upon free phenol present.

Applying the method to the filtrate after precipitation of uric acid gave the results shown in Table V.

## SUMMARY.

1. A method is described for the determination of free and conjugated phenols in the blood, based upon protein precipitation by tungstic acid, removal of uric acid by  $ZnCl_2$  and  $Na_2CO_3$ , and colorimetric determination of phenols by a modification of the older procedure of Folin and Denis.

2. The determination is made upon the common filtrate of Folin and Wu and permits of combination with the uric acid method of Morris and Macleod, minimizing the amount of material necessary.

3. The empirical factors representing the colorimetric relationship of phenol, resorcinol, and *o*- and *p*-cresol are redetermined by this method.

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## CHEMICAL FACTORS IN FATIGUE.

### II. FURTHER CHANGES IN SOME OF THE BLOOD CONSTITUENTS FOLLOWING STRENUOUS MUSCULAR EXERCISE.

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(Received for publication, March 27, 1923.)

In the first paper of this series the author gave evidence to show that both uric acid and sugar increase in the blood following a short strenuous period of exercise (1). In one case which was kept under observation for 2 hours following exercise the uric acid was found to increase steadily during that time.

To investigate this point further, and to extend it to include other constituents than uric acid, nine men were subjected to the same sort of exercise as in the earlier work; *i.e.*, running rapidly up and down stairs for about 10 minutes, or until almost completely exhausted. Four blood samples were taken in the usual way from an arm vein; one immediately before the exercise, one immediately after, another  $\frac{1}{2}$  hour later, and the last  $1\frac{1}{2}$  hours after the exercise. Uric acid was determined in these samples, usually by the recent method of Benedict (2), and in a few cases by the method of Morris and Macleod (3). Sugar was determined by the modified Folin-Wu method (4); chlorides by the Whitehorn method (5); amino-acid N by Folin's method (6); and free and conjugated phenols by a method recently suggested by the present author (7). All determinations were done in duplicate or triplicate.

The results given in Table I show two significant facts: (1) Uric acid although sometimes increasing but slightly following a short period of exercise, continues to increase for some time thereafter; (2) sugar, increasing decidedly after such a period of exercise,

rapidly returns to normal again, and has generally fallen to a subnormal level at the end of 1½ hours.

TABLE I.\*

Subject and sample.	Uric acid.		Sugar.	Chlo- rides.	Amino- acid N.	Phenols.	
	Benedict method.	Morris- Macleod method.				mg.	mg.
	mg.	mg.				mg.	mg.
1 a†	4.9	4.2	109		7.3		
b	5.4	4.9	117		7.3		
c	5.5	5.1	110		7.6		
d	5.8	6.5			6.9		
2 a	3.5	3.5	99	264			
b	3.6	3.6	107	281			
c	4.0	3.7	100	265			
d	4.0	4.0	77	279			
3 a	3.8		91	305			
b	3.8		121	308			
c	4.2		95	303			
d	3.8		85	304			
4 a		3.8	166	290	6.4		
b		3.9	181	313	6.5		
c		4.5	173	297	6.5		
d		4.8	133	297	8.0		
5 a	4.0	3.6		306	7.0		
b	4.2	3.7		311	6.9		
c	4.3	3.8		307	7.0		
d	4.3	3.9		309	6.6		
6 a	5.3		97	296		2.51	0.12
b	5.4		126	297		2.51	0.60
c	5.9		100	299		2.43	0.80
d	6.2		90	301		2.44	0.60
7 a	2.3		98	313	6.7	1.80	0.17
b	2.7		161	315	5.7	1.78	0.29
c	2.9		105	314	6.5	1.72	0.37
d	3.1		93	310	6.5	1.84	0.22

\* All values given per 100 cc. of blood.

† In Tables I and II, Sample a was taken immediately before the exercise; Sample b, immediately after the exercise; Sample c, ½ hour after the exercise; Sample d, 1½ hours after the exercise.

TABLE I—*Concluded.*

Subject and sample.	Uric acid.		Sugar.	Chlo- rides.	Amino- acid N.	Phenols.	
	Benedict method.	Morris- MacLeod method.				Free.	Conju- gated.
8 a	3.6		132	302	7.3	1.80	0.36
b	3.6		127	304	7.2	1.92	0.24
c	3.9		124	298	7.1	1.91	0.21
d	4.0		100	303	7.2	2.05	0.19
9 a	4.2			307	8.9	2.21	0.23
b	4.3			307	6.0	2.12	0.23
c	4.4			303	5.7	2.11	0.24
d	4.8			307	5.5	2.02	0.41

The high initial sugar value for Subject 8 was probably the result of an emotional reaction. Difficulty was encountered in obtaining this sample, with more pain than usual.

The other constituents studied would seem to have undergone no significant change. Free phenols do not seem to have been

TABLE II.\*  
*Average Changes of Sugar, Chlorides, and Uric Acid.*

Sample.	Uric acid by Benedict method.	Sugar.	Chlorides.
		mg.	mg.
a	0	0	0
b	+0.15	+21	+7
c	+0.44	+ 2	+0
d	+0.55	-17	+3

\* All values given per 100 cc. of blood.

concerned, although in one case at least the change in conjugated phenols is well outside the experimental error, even though these results are obtained by difference. The values for amino-acid N are apparently somewhat erratic in a few cases. It is doubtful if the method is sufficiently accurate for this type of work.

There would seem to be a slight tendency for chlorides to increase immediately following the exercise. Experience has shown that the limit of error in the method used is not more than 2 per cent. In at least two cases the increase amounted to much

more than this and not once was a decrease found. Such an increase might, of course, arise from a slight concentration of the blood.

In Table II, the average changes are summarized for those constituents which may be considered of most importance.

#### SUMMARY.

Determinations of the following constituents were made in the blood of nine men before and after a short period of strenuous muscular exercise: uric acid, sugar, amino-acids, chlorides, and free and conjugated phenols. Samples were taken at intervals up to  $1\frac{1}{2}$  hours following the exercise. Uric acid was found to increase continuously over the period of observation; sugar increased immediately following the exercise, but fell below normal at the end of  $1\frac{1}{2}$  hours. None of the other constituents altered significantly with the possible exception of a slight increase in chlorides immediately following the exercise.

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## A TRAP FOR THE VAN SLYKE GAS ANALYSIS APPARATUS.

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(Received for publication, March 10, 1923.)

A simple and effective trap is suggested for use with the Van Slyke apparatus for gas analysis. It is needed because of the difficulty of obtaining correct determinations when an apparatus is newly set up or is taken apart and cleaned. Small bubbles of air held along the sides of the glass or rubber tubing are dislodged gradually. Further, there is always the danger of a leak where the rubber tubing is attached to the glass.

The principle and design are clear from Fig. 1. The trap consists of a tube with a stop-cock parallel to the long axis of the blood pipette and connected to it by an oblique side arm. It is sealed to the apparatus at *A*. Any gas or fluid from the tubing is trapped, *D*, and can be removed by means of the stop-cock *E*. In giving directions to the glass blower, specify that *B* should be sharp and in a higher, horizontal plane than *C*.

The trap can be attached to the fine bore apparatus or the constant volume apparatus and can be used with a mechanical shaker.

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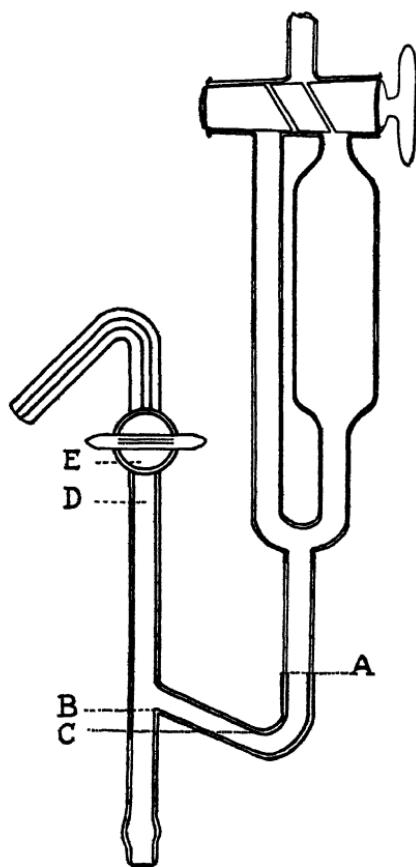


FIG. 1.

## STUDIES IN NARCOSIS.

### I. ETHER ANALYSIS.\*

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(Received for publication, March 14, 1923.)

A simple and reliable method for the determination of ether in expired air is still needed. Some methods described are laborious and some are inaccurate. The method of Kochmann and Strecker (1) seems the most simple. It depends upon the absorption of ether by alcohol and absorption of alcoholic vapors by water. Such a procedure affects subsequent analysis in view of the fact that carbon dioxide is about three times as soluble in alcohol as it is in water (2). The methods of Nicloux (3) and Spenzer (4) were unsuitable for our purpose. Nicloux's method depends upon the oxidation of ether in a sulfuric acid-chromate mixture. The method was found inaccurate, an observation also noted by Madelung (5) and Kochmann and Strecker (1). The method used by Spenzer (4), Rosenfeld (6), and later by Madelung (5), consists of decomposition by combustion according to the principle employed in the elementary organic analysis. Such a procedure is too cumbersome when many analyses are desired.

As previously reported (7), the principle of the method depends upon the absorption of ether by concentrated sulfuric acid by which absorption occurs quickly and completely and the procedure in no way interferes with a subsequent analysis of carbon dioxide and oxygen. After the utility of the method was demonstrated it was found that Horwitz (8), upon the suggestion of Kunkel, made use of the same principle and that the principle is applied in certain industrial processes.

\* A preliminary communication on this subject was read at the Toronto meeting of the Society for Pharmacology and Experimental Therapeutics, December 29, 1922.

*Method.*

The Guthrie analyzer (9) was provided with two additional absorbers, each containing 150 cc. of concentrated sulfuric acid covered with liquid petrolatum to exclude atmospheric moisture. Strong calcium chloride solution slightly acidified with hydrochloric acid and colored with methyl orange was used as a displacing liquid. No vapor of hydrochloric acid is given off in measurable quantities if the calcium chloride does not exceed 55 per cent. The solution is prepared as follows: 55 gm. of desiccator calcium chloride, without correction for moisture, are dissolved in water, filtered, and made up to 100 cc. A little methyl orange is added to color and hydrochloric acid to acidify, just sufficiently to change the color from yellow to red. The addition of acid is necessary on account of small amounts of calcium oxide present in commercial preparations.

The procedure is the same as for an ordinary air analysis (9) except that ether is removed first. Two to three displacements are adequate for complete absorption. In practice, however, six to ten displacements are made to facilitate cooling of the gas to room conditions. The purpose of the second absorber is for the storage of nitrogen. After ether is removed an approximately equivalent volume of nitrogen is added so that the oxygen readings will fall on scale. A pyrogallate solution is obviously the best for the storage of nitrogen. In practice concentrated sulfuric acid has been found suitable, particularly if the nitrogen of each analysis is pushed into the storage absorber. For long continued storage it is kept in the pyrogallate absorber.

In view of the fact that the results of some of the control experiments reported in this paper fall within range of exactness uncommon for instruments of this character it seems desirable to describe the conditions under which they were procured. The two most disconcerting factors of such instruments are incomplete drainage and variation of temperature.

1. *Drainage.*—The drainage factor can be controlled in a short burette provided it is kept clean and the drainage time is carefully taken. A drainage period of 3 minutes, as described by Guthrie (9), was found most suitable. At this time, rate of drainage has decreased sufficiently to enable one to read accurately and is the

last minute period in which drainage is constant. For best results the drainage period should begin at the instant that the bulb is lowered. The reading at the end of the period should not exceed 3 minutes by more than 10 seconds. In a test with the solution in use drainage lacked 0.06 cc. of being complete in 3 minutes at the total volume and carbon dioxide levels while at the oxygen level it lacked only 0.02 cc. This error becomes compensated and at times exceeded in view of the unavoidable heating which has occurred when the oxygen reading is taken. With suitable

TABLE I.  
*Rate of Drainage in Analyzer Burette.*

Drainage time. min.	Total volume level.				CO <sub>2</sub> level.		O <sub>2</sub> level	
	Test I.	Test II.	Test III.	Test IV.	Test I.	Test II.	Test I.	Test II.
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
1	0	0	0	0	0	0	0	0
2	0.09	0.09	0.09	0.09	0.08	0.08	0.03	0.03
3	0.03	0.03	0.03	0.03	0.03	0.03	0	0
4	0.03	0	0.03	0.02	0.01	0.01	0.01	0.01
5	0.01	0.01	0.01					
6	0.01							
7	0							
8	0.01							
9	0							
20	0	0.05	0.02	0.05	0.05	0.05	0.01	0.01
Total drainage.....	0.18	0.18	0.18	0.19	0.17	0.17	0.05	0.05
Drainage in 3 min.....	0.12	0.12	0.12	0.12	0.11	0.11	0.03	0.03
Incomplete drainage in 3 min.....	0.06	0.06	0.06	0.07	0.06	0.06	0.02	0.02

precautions, therefore, theoretical analyses of outside air are obtained with surprising frequency.

Results of an experiment illustrating rate of drainage are recorded in Table I. After room air was drawn into the burette the rubber connection to the leveling bulb was clamped, the burette remaining open to atmospheric air. Drainage readings were taken at various levels, at minute intervals and finally at a time when drainage was known to be complete, usually after 20 minutes. The first reading always occurred after a drainage of 1 minute.

*2. Temperature.*—Analyses were always made under stabilized room conditions. Carelessness in this regard leads to inaccurate results even with an instrument provided with a compensator such as is the Haldane. With the latter instrument it has been observed repeatedly that compensation was most complete for slight variations in temperature only; *i.e.*, when compensation was least necessary. There seems to be a tendency towards carelessness with such instruments because of failure to realize the limits of compensation. On the other hand, with an instrument not so provided, the operator realizes that every precaution must be taken against temperature variations so that very good results can be obtained with a simpler instrument.

TABLE II.  
*Test Showing Suitability of Room Conditions.*

Test No.	Volume. cc.	Absorber.	Displacement.
1	39.78	NaOH	4
2	39.79	H <sub>2</sub> SO <sub>4</sub> , No. 1	3
3	39.79	H <sub>2</sub> SO <sub>4</sub> , No. 2	3
4	39.79	NaOH	4
5	39.79	H <sub>2</sub> SO <sub>4</sub> , No. 1	3
6	39.80	H <sub>2</sub> SO <sub>4</sub> , No. 2	3
7	39.80	NaOH	4

One may determine whether room conditions are suitable by passing a non-absorbable gas, such as nitrogen, into the various absorbers as a preliminary procedure. The determinations should agree within 0.01 to 0.02 cc. A test of such character is recorded in Table II. In this particular case a mixture of oxygen and nitrogen was used since oxygen determinations were not desired on that day.

*3. Calcium Chloride as a Displacing Liquid.*—After it was established that sulfuric acid was a satisfactory absorbant for ether it was necessary to determine the effect of treating carbon dioxide and oxygen with sulfuric acid in a similar manner as might occur in routine analyses. Expired air was stored in a 17 liter bottle under pressure by forcible expiration through a tube placed in the mouth. Such a bottle will furnish 2 liters of air of constant composition, without the use of a displacing

liquid for its transfer to the analyzer. Samples were analyzed for carbon dioxide and oxygen both before and after exposure to sulfuric acid. With the use of acidulated water in the instrument it was found that about 0.30 per cent of the sample was absorbed after exposure to sulfuric acid, Table III. Later tests showed that the loss was due to absorption of carbon dioxide by the displacing liquid.

To determine the efficiency of a 55 per cent calcium chloride solution as a displacing liquid, carbon dioxide samples of uniform composition were analyzed with the Haldane analyzer, using mercury, and with the Guthrie apparatus, using acidulated water, and acidulated water saturated with the same gas before the first test and a 55 per cent calcium chloride solution. The results tabulated in Table IV show that with the use of calcium chlo-

TABLE III.  
*Results Showing Loss of CO<sub>2</sub> When Displacing Liquid Consists of Acidulated Water.*

CO <sub>2</sub> loss after H <sub>2</sub> SO <sub>4</sub> treatment. per cent	Absorption by NaOH. per cent	Total CO <sub>2</sub> . per cent	O <sub>2</sub> per cent
0.30	2.63	2.93	17.76
	2.90	2.90	17.75
	2.87	2.87	17.75
0.37	2.55	2.92	17.77
0.30			
0.32	2.63	2.95	17.79

ride in the Guthrie instrument determinations are nearly as accurate as with the Haldane instrument. It further shows that acidulated water alone gave inaccurate results, but that such a solution saturated with carbon dioxide gave satisfactory results, a conclusion verified in our student laboratory. Such saturation is required for each analysis as is indicated by the progressive decrease of percentages in which only a single saturation was employed.

4. *Completeness of Ether Removal from Expired Air Gases.*—After it was demonstrated that a calcium chloride solution, under conditions as specified, absorbed very little carbon dioxide, it was necessary to determine whether there was any absorption by sulfuric acid. Samples of carbon dioxide and air mixtures of

uniform composition were analyzed for carbon dioxide. Other samples were analyzed after exposure to sulfuric acid, while others were analyzed for carbon dioxide after the samples were passed over liquid ether and the vapors subsequently absorbed by exposure to sulfuric acid. The latter procedure was to test the completeness of ether absorption in the presence of carbon dioxide. The results are essentially identical, showing that no carbon dioxide is absorbed by sulfuric acid, Table V. The

TABLE IV.  
*Comparison of CO<sub>2</sub> Analyses by Various Procedures and Instruments.*

Haldane.		Guthrie	
Mercury.	Acidulated H <sub>2</sub> O.	Acidulated H <sub>2</sub> O + single CO <sub>2</sub> saturation.	55 per cent CaCl.
per cent	per cent	per cent	per cent
3.12	2.91	3.10	3.10
3.14		3.07	3.12
3.12		3.05	3.13
		3.02	3.12
3.13		3.00	3.10
			3.11
Average.....	3.13	3.05	3.11

TABLE V.  
*Showing Completeness of Ether and CO<sub>2</sub> Absorption in Each Other's Presence.*

CO <sub>2</sub> by usual analysis.	CO <sub>2</sub> analysis after treatment with sulfuric acid.	CO <sub>2</sub> analysis after removal of added ether.
per cent	per cent	per cent
2.96	2.98	2.94
3.02	2.98	3.00
2.97	2.97	2.97
Average...2.98	2.98	2.97

greater variation of individual results with the ether samples was presumably caused by irregular temperature variations such as cooling from ether evaporation and subsequent heating from ether absorption. At the date of this experiment the temperature factors of the method were not fully appreciated.

The efficiency of ether removal by sulfuric acid was also tested upon atmospheric air from which added ether was success-

fully removed as indicated by corresponding results obtained with control air, Table VI. These results, therefore, show that ether is completely removed from gases found in expired air by treatment with sulfuric acid without affecting the subsequent analysis.

*5. Limits of Ether Absorption by Sulfuric Acid.*—It seemed desirable to determine how much ether may be absorbed by sulfuric acid before absorption becomes incomplete. Two 50 cc. graduated cylinders were arranged in series with a 250 cc. bottle with wash bottle connections. The first cylinder contained 22 cc. of ether, the second 21 cc. of concentrated sulfuric

TABLE VI.  
*Results Showing the Successful Removal of Ether from Outside Air.*

CO <sub>2</sub> per cent	O <sub>2</sub> per cent	Ether. per cent	Remarks.
0.05	20.90	2.53	Outside air with ether.
0.00	20.94	2.50	
0.03	20.89	2.54	
0.00	20.96	2.48	
Average.0.02	20.92	2.51	
0.00	20.88		Outside air.
0.05	20.90		
0.00	20.96		
0.025	20.92		
Average.0.018	20.91		

acid, and the bottle served as a safety and sampling device. Air was drawn through by mouth suction to detect ether vapors and the content of the bottle was analyzed at intervals. The volumes of liquids in the cylinders were determined from time to time. The results, tabulated in Table VII, show that absorption is complete except after the volume of acid has nearly doubled. In view of the fact that the volume of sulfuric acid increases with absorption of ether, experiments were performed as before, but arranged with two cylinders of sulfuric acid. Drawing ether vapor through them, the volume of the second acid absorber was not increased until that of the first had been

nearly doubled. Inasmuch as the absorber on the analyzer contains 150 cc. of acid, and since 1 cc. of ether is equivalent to more than 200 cc. of vapor, the absorber would absorb completely until 30 liters of vapor had been taken up. In routine analysis less than 4 cc. of vapor are found in expired air samples. Hence, on that basis 7,000 analyses could be made before absorption became incomplete.

*6. Determination of Number of Displacements Required.*—In order to determine the number of displacements into the acid required to insure complete absorption, 3 to 4 per cent ether mixtures in air were introduced into the analyzer. Determinations were made after a variable number of displacements. Constancy of readings was taken to indicate complete absorption.

TABLE VII.  
*Results of Aspirating Ether through Sulfuric Acid.*

Volume in ether cylinder. cc.	Volume in H <sub>2</sub> SO <sub>4</sub> cylinder. cc.	Ether detected by taste.	Unabsorbed ether by analysis. per cent
22	21	None.	
19	23	"	None.
18	24	"	
17	25	"	
15	27	"	None.
11	30	"	
9	32	"	None.
2	39	Faint taste.	0.42

It was found that after two or three displacements further displacements did not alter the readings, as is illustrated in Table VIII. However, later tests revealed that ether concentrations of 8 to 10 per cent require six displacements into the acid before concordant results are obtained, Table IX.

*7. Heating of Gas during Absorption.*—The following tests were applied to determine whether the greater number of displacements necessary for higher concentrations of ether was a temperature factor or one of slower absorption. A variable number of displacements were made employing one or both absorbers on vapor mixtures of 8 to 10 per cent. It was found that when the displacements were divided between the two absorbers, three total displacements were equivalent to six made

in one absorber. This led to the belief that although three displacements were adequate for complete absorption an additional number was required to facilitate cooling of the gas heated during absorption. The correctness of this belief was later verified. By the employment of only three displacements in one absorber and allowing the gas to cool spontaneously for an hour, it was found that the remaining gas did not contain ether (Test VII, Table IX).

TABLE VIII.  
*Number of Displacements Required for Complete Absorption.*

Test No.	Readings.	Displacements.
		cc.
I	40.00	0
	38.28	1
	38.22	2 more.
	38.22	10 "
II	39.98	0
	38.26	3
	38.26	10 more.
III	40.01	0
	38.50	2
	38.50	10 more.

A similar difficulty was experienced by Horwitz (8), employing ether concentrations of 38 to 84 per cent. He concluded that absorption required 38 to 75 minutes. Presumably, he was led to this conclusion by the heating of the gas which required a long time to cool. The heat of reaction of ether and sulfuric acid is comparable to the familiar reaction of water and sulfuric acid.

#### SUMMARY.

A method for the analysis of ether in expired air is described, together with notes on air analysis employing an aqueous analyzer from which results are obtained that compare favorably with those obtained from a mercury instrument.

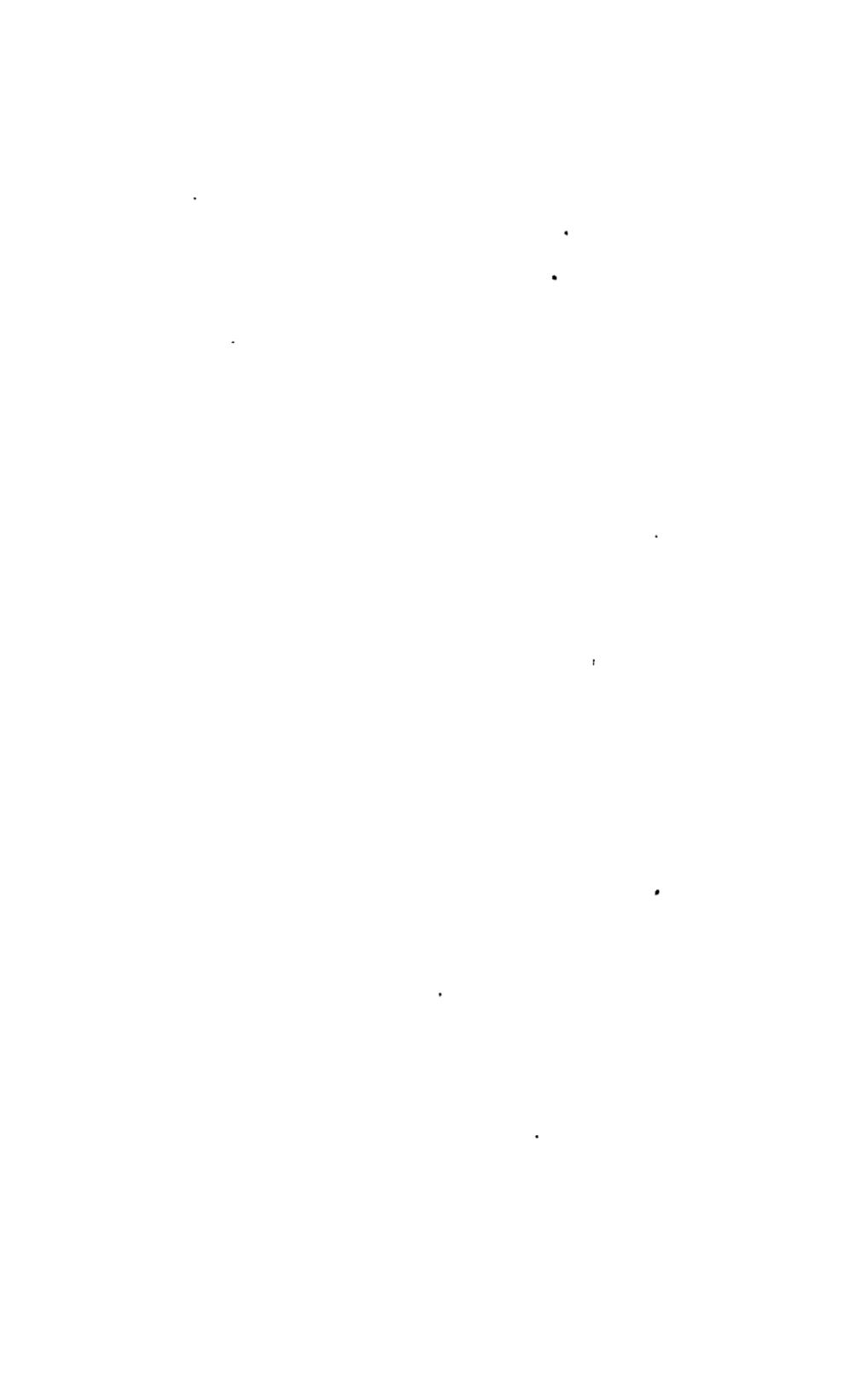
TABLE IX.  
*Results Illustrating the Heat of Absorption.*

Test No.	Volume of gas. cc.	Displacements in Absorber I.	Displacements in Absorber II.
I	39.78		
	36.37	3	
	36.28		3
	36.28	3	
II	40.00		
	36.50	3	
	36.42	3	
	36.42	3	
III	39.72		
	36.17	1	2
	36.17	3	
	36.17		3
IV	39.80		
	36.28		3
	36.22	3	
	36.21		3
V	39.79		
	36.32	2	1
	36.32	3	
	36.31		3
VI	39.97		
	36.45	6	
	36.44		3
VII	39.83		
	36.32	3	
	36.28	Volume after an hour.	
	36.28	3	

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## STUDIES IN NARCOSIS.

### II. A METHOD FOR THE DETERMINATION OF THE RESPIRATORY EXCHANGE DURING ETHER NARCOSIS.\*

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In 1917 Guthrie (1) reported certain shock experiments in which respiratory exchange was studied on animals in ether narcosis. At that time a suitable method for the separation of ether vapor from the expired air was not available. Since then such a method has been devised (2). The present report deals with a description of the application of the method to studies of respiratory exchange in anesthetized animals, with a preliminary report of results upon animals in various stages of reduction.

#### *Behavior of Ether in a Spirometer.*

Ether confined in a spirometer does not remain uniform in composition due to its solubility in water, condensation, tendency to stratify, etc. A loss of ether does not affect the results of respiratory exchange if uniformity of mixture is maintained during the sampling period. However, an effort was made to prevent as much loss as possible in order to increase the usefulness of the method for the determination of the concentration of ether exhaled.

1. *Solubility in Water.*—In a spirometer of 15 liter capacity with water over the entire cross-section of the chamber, ether is lost appreciably in a short time. In one experiment consecutive

\* A preliminary communication on this subject was read at the Toronto meeting of the American Physiological Society, December 27, 1922. An abstract of this paper was published in the American Journal of Physiology (Kruse, T. K., *Am. J. Physiol.*, 1923, lxiii, 398).

analyses, spaced 15 minutes apart, yielded the following concentrations; 11.50, 10.02, and 9.98 per cent, respectively. Over a 24 hour period there was a loss of 66 to 81 per cent of ether, while the loss of carbon dioxide and oxygen was only 12 to 16 per cent and 0.6 to 1 per cent, respectively.

In spirometers of 75 to 100 liter capacities, so constructed that the water is confined to a sealing space, loss by solubility is slight after the initial change has occurred. For instance, in a spirometer freshly filled with ether vapor, when mixed before each test to insure uniformity of concentration, consecutive analyses spaced 20 minutes apart yielded 11.25, 11.24, and 11.20 per cent, respectively. In another experiment, Table I, the concentration diminished from 8.83 to 8.23 per cent. In this experiment the spirometer had been used for ether storage for a number of days. It is very evident that the loss of ether from this source in a suitable spirometer is without significance in studies of this character.

*2. Stratification.*—The extent of stratification is recorded in Tables I and II. To insure uniform mixture a hand syringe bulb was attached to the spirometer so that air was withdrawn from the top of the bell and forcibly pumped through a movable but sealed tube extending for a foot into the bell. 50 to 100 complete discharges are adequate for mixing 40 liters of gas. In Table I the spirometer was filled with air and ether vapor and mixed at once by 100 discharges of the hand pump. Ether and carbon dioxide concentrations were determined at intervals. The carbon dioxide percentages are calculated upon the volume after absorption of ether and, therefore, are not dependent upon ether variations and serve to control the analyses. In Table II there is a similar experiment recorded with the exception that the ether had stratified from the previous day and carbon dioxide analyses were omitted. It is seen from the results that within 2 hours of collection the concentration may vary, due to stratification, from 8.84 to 8.67 per cent, Table II, or from 8.63 to 8.46 per cent, Table I, with ether concentrations of 8 to 9 per cent, if sampled from the middle portion of the bell. When the gas is mixed by pumping before sampling, ether concentrations may be kept uniform, 11.35 a.m. and 12.07 p.m., Table I, and 10.28, 10.45, and 11.01 a.m., and 1.46 and 2.01 p.m., Table II. Manipulation

of the bell also tends to mix the gas, but less thoroughly. This is seen when consecutive analyses of an unmixed supply yielded

TABLE I.  
*Showing Behavior of Ether Vapor in Spirometer.*

Date.	Time.	Ether.	Carbon dioxide.*	Source.	No. of pumps.	Remarks.
1922	p.m.	per cent	per cent			
July 7	4.00				100	Refilled spirometer.
	4.04	8.83	3.00	Middle.		
	4.17	8.75	3.03	"		
	4.30	8.71	2.97	"		
	4.45	8.58	3.02	"		
Average.....		8.72	3.00			
	a.m.					
July 8	9.20	8.00	2.94	Top.		
	9.34	8.30	2.94	"		
	9.51	8.30	2.94	"		
Average.....		8.20	2.94			
	10.04	8.40	3.00	Middle.		
	10.20	8.49	3.02	"		
Average.....		8.45	3.01			
	10.45	8.63	2.96	Middle.	100	
	11.00	8.46	2.94	"		
	11.20	8.48	2.95	"		
	11.35	8.56	2.96	"	30	
	p.m.					
	12.07	8.55	2.94	"	100	
Average.....		8.54	2.95			
	a.m.					
July 12	9.02	8.09	2.77	Middle.	50	
	9.20	8.21	2.78	"	50	
	9.48	8.28	2.78	"		

\* Calculated upon volume after ether absorption.

increasing concentrations, 9.20, 9.34, 10.04, and 10.20 a.m., Table I, and 9.02, 9.14, and 9.32 a.m., and 1.02, 1.17, and 1.30 p.m., Table II. After mixing, stratification begins at once as is

illustrated in Table I in which consecutive analyses spaced 15 minutes apart yielded 8.83, 8.75, 8.71, and 8.58 per cent, respectively. This is also seen at times when concentrations just after mixing are lower than samples collected a little later and analyzed upon the following day, Table III, Samples I and III. In view of the fact that carbon dioxide and oxygen percentages are based upon the volume after ether absorption, variations of ether per cent will affect only the total volume correction of the experimental period. In such experiments, were mixing neglected

TABLE II.  
*Showing Behavior of Ether Vapor in Spirometer.*

Time.	Ether.	Source.	No. of pumps.	Remarks.
a.m.	<i>per cent</i>			
9.02	8.23	Top.		Mixture undisturbed for 18 hrs.
9.14	8.53	"		
9.32	8.64	"		
9.45	8.72	Middle.		
10.00	8.70	"		
10.12	8.77	"		
10.28	8.84	"	50	Uniformity due to mixing.
10.45	8.84	"	50	
11.01	8.83	"	50	
p.m.				
1.02	8.67	"		
1.17	8.68	"		
1.30	8.72	"		
1.46	8.81	"	50	Uniformity due to mixing.
2.01	8.81	"	50	

the error would be less than 40 to 100 cc. in a volume of 40 liters, a high determination of an average experimental period. Such a volume is less than can be read with accuracy on the usual spirometer. However, since the type of respiration sometimes changes during a test and, therefore, the concentration of ether, it seemed desirable to mix the gas before sampling even though the variation usually is quite small.

*3. Absorption by Rubber.*—Since ether vapor is rapidly taken up by rubber this material should be reduced to a minimum in apparatus containing ether vapor. White rubber absorbs less

ether than the black gum rubber. In an experiment in which 2 feet of gum tubing were used for about 2 hours this characteristic was very apparent. This tube, after expelling all the contained gas, was layed aside. A few minutes later, upon inhaling through the tube, a suffocating concentration of vapor was found. Air was then blown through the tube to expel all vapor present. A few minutes later the contents of the tube were analyzed and ether vapor was present in 9.5 per cent concentration. A similar experiment performed with white rubber gave a concentration of ether vapor less than 1 per cent.

4. *Sampling.*—Whenever possible analyses were made directly from the spirometer. However, under experimental conditions complete analyses had to be delayed and, therefore, required some suitable method of storing samples. The following method and apparatus were found suitable. The 500 cc. glass bottle sampler employed by Guthrie (3) was used. Acid water was used for displacement. 4 to 5 additional liters were pushed through the sampler after complete water displacement had occurred in order to displace the first portion which usually has lost some gas by solubility in water. The rubber connections were tightly clamped and the sample was set aside for analysis. Up to 24 hours after sampling good results were obtained, provided displacement from the sampler to the analyzer was made with 55 per cent of desiccator calcium chloride solution (2).

Table III shows that collected samples taken by this method give as good results 24 hours after collection as when taken directly from the spirometer at the time of the experiment. With the employment of concentrated calcium chloride solution consecutive analyses are usually nearly identical for all determined gases. A 500 cc. sampler already containing about 150 cc. of displacing fluid consisting of calcium chloride can be used for still another day without introducing an excessive error in the oxygen or carbon dioxide determination. The ether lost by this time is somewhat greater as is illustrated in the following percentages found, 4.65 to 4.33, 3.85 to 3.51, 6.28 to 5.69, 6.83 to 6.48. Inasmuch as the ether concentrations are essential for the correction of total ventilation volume and the possible error is less than 1 per cent it is obvious that by this method analyses may be made within several days after the experiment without introducing prohibitive errors.

Table IV illustrates a similar experiment. Consecutive analyses are essentially identical for oxygen and carbon dioxide but for ether the variations are greater. This was in part due to the fact that analyses at the time of the experiment and sampling for later analyses were spaced farther apart after mixing than occurred in Table III and to the fact that in the latter experiment a loose piece of glass tubing was dropped in the bottle before sampling to insure a thorough mixing of the gas by shaking the

TABLE III.  
*Comparison of Analyses of Samples Taken Directly from Spirometer, 24 and 48 Hours after Sampling.*

Sample No.	Ether.		CO <sub>2</sub>		O <sub>2</sub>		Remarks.
	Test 1.	Test 2.	Test 1.	Test 2.	Test 1.	Test 2.	
	per cent	per cent	per cent	per cent	per cent	per cent	
I	4.59		2.62				From spirometer.
	4.65		2.61		18.58		" sampler after 24 hrs.
	4.33		2.53		18.59		48 hrs. after sampling.*
II	3.85		2.07				From spirometer.
	3.85	3.85	2.07	2.06	18.87	18.86	" sampler after 24 hrs.
	3.51		2.00		18.88		48 hrs. after sampling.*
III	6.18		3.22				From spirometer.
	6.28		3.23		17.31		" sampler after 24 hrs.
	5.69		3.22		17.35		48 hrs. after sampling.*
IV	6.84	6.83	4.53	4.54	16.10	16.02	From spirometer.
	6.83		4.54		16.07		" sampler after 24 hrs.
	6.48		4.51		16.04		48 hrs. after sampling.*

\* Sampler contained 150 cc. of CaCl<sub>2</sub> solution for 24 hours.

bottle before each test. This was accomplished by holding the bottle horizontally and shaking with a rotary motion. The absence of this procedure in Table IV is apparent in the ether column in that the second analysis is higher than the first. In no case, however, are the results sufficiently discordant to affect conclusions in studies of this character.

#### *Method of Procedure.*

After the above facts were determined and the technique for accurate determinations established the method was applied to animals under experimental conditions. Dogs were anes-

thetized with ether and arranged to record blood pressure and respiration. Ether was administered by tracheal cannula attached to an automatic device provided with valves to separate the expired from the inspired air (4). The valves were always tested to insure absence of leaks. The expired air was led by a glass tube to either a 75 or 100 liter spirometer. The collected gas of the initial period was always expelled to wash out connections and the dead air space of the spirometer. The collection period usually lasted 10 minutes. Blood pressure and respiration

TABLE IV.

*Comparison of Analyses of Samples Taken Directly from Spirometer and 24 Hours after Sampling.*

Sample No.	Ether.		CO <sub>2</sub>		O <sub>2</sub>		Remarks.
	Test 1.	Test 2.	Test 1.	Test 2.	Test 1.	Test 2.	
	per cent	per cent	per cent	per cent	per cent	per cent	
I	6.97						Taken from spirometer.
	6.86		1.89	1.87	18.69	18.65	24 hrs. later from sampler.
II	8.01		2.24				Taken from spirometer.
	7.20	7.47	2.29	2.24	18.14	18.10	24 hrs. later from sampler.
III	6.48		2.17		18.10		72 " after sampling.
	1.20		2.05				Taken from spirometer.
IV	1.23	1.25	2.07	2.08	18.57	18.58	24 hrs. later from sampler.
	6.41		1.42				Taken from spirometer.
V	5.84	6.09	1.39	1.39	19.42	19.45	24 hrs. later from sampler.
	6.58		1.67				Taken from spirometer.
VI	5.79	6.03	1.66	1.64	18.98	19.02	24 hrs. later from sampler.
	1.05						Taken from spirometer.
VII	1.08	1.08	1.70	1.67	19.42	19.46	24 hrs. later from sampler.
	0.86		1.34				Taken from spirometer.
	0.90		1.31	1.37	19.69	19.71	24 hrs. later from sampler.

tracings were taken during the collection period. The gas in the spirometer was pumped 100 times with the hand syringe bulb. The volume and temperature were recorded. Analyses for ether and carbon dioxide were made at once. A 500 cc. sample was then taken by displacement with acid water. After all the water was out 4 to 5 additional liters were pushed through the sampler. These analyses were usually made on the following day. In this manner the respiratory exchange was studied on dogs in various stages of reduction, with or without further administration of

ether. Of course, in no instance was an animal permitted to regain consciousness. The oxygen and carbon dioxide percentages are based on the volume after ether absorption. A correction was not made for the ether retained by the body. Inasmuch as the changes in respiratory exchange were so much greater than the possible error from this source its determination was not made.

*Results with the Method.*

Figs. 1 and 2 illustrate the nature of the results obtained. The dotted lines in Fig. 1 indicate periods when ether administration was discontinued. Calculations are based on 10 kilo dogs. The actual weights varied from 10 to 12 kilos. In Fig. 2 average determinations are represented for the first 2 to 3 hour period arranged according to ether concentrations exhaled. Inasmuch as concentrations of exhaled ether increase with the intensity of administration one may assume that such determinations indicate the intensity of etherization. Tables V, VI, VII, and VIII represent abbreviated protocols of those experiments which were free from obvious technical errors. The observations may be summarized as follows:

1. In moderate to deep anesthesia, extending over many hours the oxygen consumption and carbon dioxide elimination described a fairly constant reduction curve dependent more upon duration of anesthesia than upon precise procedures. Light anesthesia showed a similar reduction curve, but at a higher level. Removal of ether markedly augmented respiratory exchange, but such increases became progressively less with the length of narcosis.
2. The sums of actual oxygen and carbon dioxide percentages in deep narcosis were as low as 19.93, and as high as 21.60 when ether was removed.
3. Carbon dioxide elimination was depressed more than oxygen consumption in deep narcosis and the reverse was true upon removal of ether. The respiratory quotients accordingly decreased, but in the terminal stage of deep anesthesia they might increase, Experiment 4 and Samples I, II, and III of Experiment 5.
4. For equal duration of narcosis, the depression of metabolism was associated with intensity of narcosis (Fig. 2).
5. In light anesthesia, the oxygen consumption exceeded carbon dioxide elimination provided the animal had not been

TABLE V.  
*Experiment 4.*  
Dog 4. Weight 11 kilos. Nov. 14, 1922.

TABLE VI.  
*Experiment 6.*



TABLE VII.  
*Experiment 6.*  
Dog 6. Weight 10 kilos. Nov. 27, 1922.

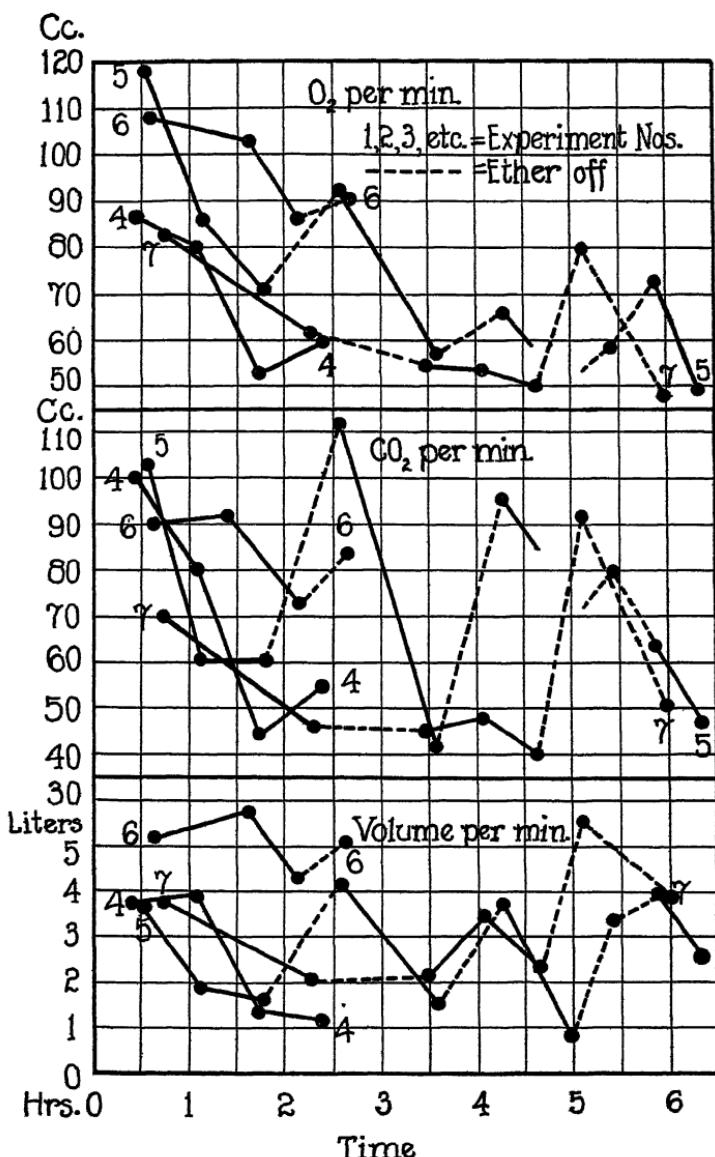


FIG. 1. Superimposed curves of Experiments 4, 5, 6, and 7 showing oxygen consumption, carbon dioxide elimination, and ventilation rates per 10 kilo dog. Experiment 6 was one of light anesthesia.







previously saturated with ether (Fig. 1), Experiment 6 and Samples III, IV, V, and VI of Experiment 7. In the latter test the animal was resuscitated after the heart had stopped beating. Artificial respiration was employed for 10 minutes before spontaneous respiration began. The test was made immediately after respiration began. After the ether had been removed for 42 minutes it had to be readministered due to return of reflexes. 5 minutes after its readministration another test was made. In both tests oxygen

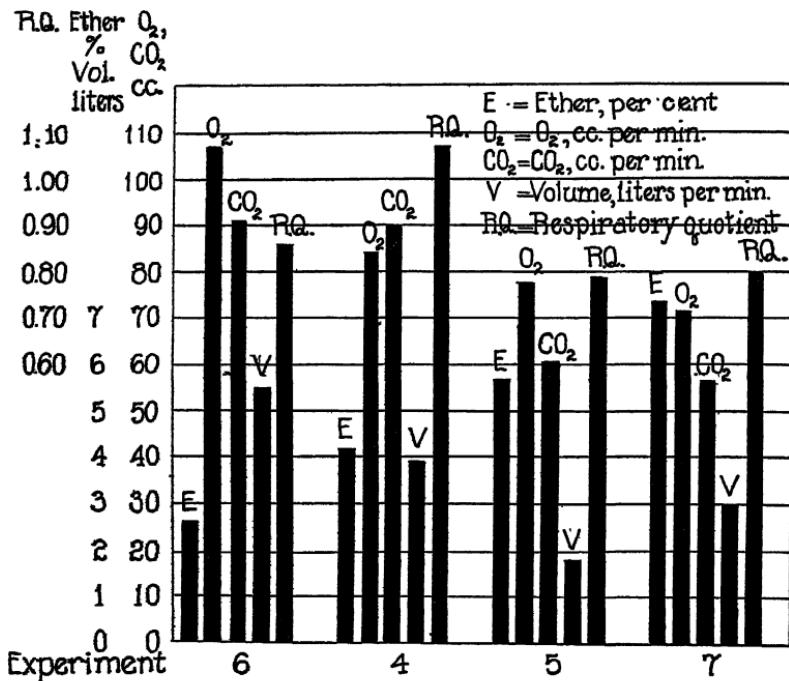


FIG. 2. Showing average results during the first 2 hours of anesthesia.

consumption exceeded carbon dioxide elimination. A little later the animal again became saturated with ether and upon its removal carbon dioxide elimination again exceeded oxygen consumption. Ventilation rates do not seem to be determining factors. The influence of ether upon the metabolism seems to involve other mechanisms. The close relationship of ether concentration exhaled to carbon dioxide eliminated is further illus-

trated in Dogs 6 and 4 (Fig. 2), in which the former dog with the higher ventilation rate eliminated the less carbon dioxide as represented by the respiratory quotients. Another illustration occurs in Dogs 5 and 7 in which the latter dog with a third greater ventilation rate eliminated the less carbon dioxide, the respiratory quotients being essentially equal.

6. Animals in a state of reduction require much less anesthetic as the reduction increases. The results of Guthrie on animals in shock are qualitatively supported by these studies. In his experiments the ether factor was only partially controlled inasmuch as a suitable method was unavailable at that time.

The precise interpretation of these results and their relation to the results of others is delayed until the series is more complete. The current interest for better anesthesia justifies the description of results though based on a limited number of experiments.

#### SUMMARY.

A method is described which is suitable for the study of the respiratory exchange in etherized animals, with a preliminary report of results obtained upon animals in various stages of reduction.

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## A NEW SULFUR-CONTAINING AMINO-ACID ISOLATED FROM THE HYDROLYTIC PRODUCTS OF PROTEIN.\*

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During an investigation of the cultural requirements of certain bacteria, the writer isolated from the hydrolytic products of casein, a fraction of material which was apparently required by hemolytic streptococci for satisfactory growth, and which was found on examination to contain a considerable amount of sulfur (1). The sulfur was apparently not in the form of cystine since it did not give the customary reactions (lead blackening and sodium nitroprusside) which are given by the sulfur in this compound. Since the participation, as growth factors under the experimental conditions, of any of the known amino-acids had been ruled out, it was thought possible that this new sulfur compound might be the factor which was being sought from the casein. An attempt was, therefore, made to isolate the material in sufficient quantity and purity to determine its chemical nature; and although the final separation in pure form proved exceedingly difficult, it was found that the sulfur compound lost its activity as a growth-inducing substance when it became even relatively pure. It is not yet clear whether this is due to some kind of chemical change in the sulfur compound, or simply to the elimination of a separate active substance during the purification. In view, however, of the general impression that there was present in protein one or more other sulfur-containing amino-acids, besides cystine, it seemed to be important to carry through the isolation of the

\* During the summer of 1922, a portion of the work on purification was carried out in the Biochemical Laboratory, Cambridge University, Cambridge, England, and the writer wishes to express his indebtedness to Prof. F. Gowland Hopkins for this courtesy, as well as for valuable advice and suggestions.

material if it could be done, as a problem of some biochemical importance with possible bacteriological bearing.

The literature on the non-cystine protein sulfur need not be extensively reviewed in this place, beyond referring to the paper of Osborne (2) in which the existence of other forms of sulfur than cystine in the protein molecule has been fairly definitely indicated.

An amino-acid, agreeing closely with the formula  $C_5H_{11}SNO_2$ , has been obtained as a result of the work. During the progress of the investigation it has been necessary to modify the original method of preparation in many ways, and each modification has resulted in some increase in the yield. However, the writer feels that the present method is by no means quantitative, and that a still greater portion of the sulfur present in protein hydrolysates than that actually isolated can be accounted for on the basis of the compound to be described. It would seem wise, however, to present this compound as a primary constituent of the protein molecule with a good deal of conservatism. It will be shown that there is considerable evidence pointing toward the preexistence of the compound, as such, and while there is no direct evidence to the contrary, the writer does not wish to state definitely that he is convinced that the amino-acid is not formed by some secondary reaction during the hydrolysis or separation. Assurance on this point may well be withheld until more work has been done on the structure and physiological properties of the compound.

The yield obtained by the method described below varies from about 1 to 2 gm. from a pound of casein; *i.e.*, from 0.2 to 0.4 per cent. This represents about ten times the quantity obtained by earlier investigations. Because of this small yield, it has been necessary to use large quantities of casein and three preparations of from 30 to 50 pounds each<sup>1</sup> have been put through. The final purification of the compound has been a matter of much difficulty because of the presence of two or three other amino-acids, particularly phenylalanine and glutamic acid, which are

<sup>1</sup> The writer is indebted to Prof. Ralph McKee of the Department of Chemical Engineering, Columbia University, to Dr. Frederick Zinsser of Zinsser & Company, Hastings-on-Hudson, New York, and to Prof. J. C. Olsen of the Brooklyn Polytechnic Institute for the use of large scale apparatus and equipment necessary for handling this quantity of material through the preliminary stages.

found in considerable quantity in the  $\text{Ba}(\text{OH})_2$  extract of the  $\text{HgSO}_4$  precipitate. While the latter acid can be separated without a great deal of difficulty by precipitation with barium hydroxide and alcohol, the phenylalanine cannot be removed by recrystallization, nor, quantitatively, by the distillation of the ethyl esters. Before the presence of phenylalanine had been recognized in the mixture, it was found that a constant composition seemed to be reached on recrystallization, and from this mixture which has since been shown to contain phenylalanine and one other unidentified impurity with the sulfur compound, the formula  $\text{C}_{11}\text{H}_{22}\text{SN}_2\text{O}_4$  was erroneously deduced, and presented, tentatively, before the Society of Experimental Biology and Medicine in New York last year (3). By the distillation, *in vacuo*, of the ethyl esters prepared from such a mixture, it developed that the ester of the sulfur compound distilled in the same fraction with that of phenylalanine, and further, that it shared the property of the latter in being relatively insoluble in water and soluble in ether. A small amount of material was, however, obtained in this preparation from the aqueous solution of the esters, which proved to be fairly pure, contaminated only with a small amount of phenylalanine, and from this preparation the formula here presented for the sulfur compound was first calculated.

A review of the earlier modifications of the method and of the various unsuccessful efforts at purification need not be presented here. The method as now used is as follows:

#### *Preparation of the Compound.*

Casein is hydrolyzed by boiling for 18 hours with six times its weight of water, and from two to three times its weight of concentrated sulfuric acid. The solution is cooled, diluted somewhat with water, and neutralized by the addition of sodium hydroxide solution, or, better, on a large scale with commercial soda ash. The neutral mixture is allowed to cool and, if necessary, is decanted from sodium sulfate which separates if the material is not sufficiently diluted before neutralization. It is then precipitated by a solution of mercuric sulfate containing 10 per cent of  $\text{HgSO}_4$  and 5 per cent, by volume, of concentrated  $\text{H}_2\text{SO}_4$ . After adding the reagent, the mixture is neutralized with strong sodium hydroxide solution, to litmus paper. The neutralization brings down a much more bulky precipitate, but it approximately doubles the yield of sulfur compound. It is essential to use care not to carry the neutralization too far, because the compound to be isolated is quite readily soluble in dilute alkali solution. After standing

over night, the precipitate is filtered by suction, or on a filter press, and the precipitate is washed, thoroughly, with water, resuspending and filtering each time, for at least three or four washings, until the greater part of the sodium sulfate and unprecipitated amino-acids have been removed. Filtration is slow and as large a filter as possible should be used. The precipitate is then extracted with hot 2 per cent barium hydroxide solution, using approximately a liter of the solution for each pound of casein represented in the original preparation. In making the extraction, the precipitate is first suspended in water and treated with hot saturated  $\text{Ba}(\text{OH})_2$  until the reaction is faintly alkaline to litmus, and then 2 per cent additional  $\text{Ba}(\text{OH})_2$  is added. The mixture is heated for  $\frac{1}{2}$  hour, either on the water bath or with a steam coil, and is then filtered by suction or on a press. It is necessary to repeat the extraction altogether about four times, in order to obtain the maximum yield, but after the first extraction, the precipitate is suspended directly in a 2 per cent  $\text{Ba}(\text{OH})_2$  solution. The united extracts are heated to about  $60^\circ$ , a solution of barium sulfide in water is added to precipitate the mercury, and sulfuric acid is added to remove the barium. After filtering, the filtrate is evaporated in an open dish, heated directly on a gas plate in front of an electric fan, to a volume of about 200 cc., for each original pound of casein. It is then freed of an excess of either barium or sulfate, and is precipitated with a mercuric chloride solution. The solution is first brought to boiling, and then a boiling saturated solution of mercuric chloride is added, using about 30 gm. of the reagent for each pound of protein in the preparation. The precipitate will begin to separate in the hot solution, sometimes as a sticky syrup, and, occasionally, as a semi-granular material, and it is allowed to stand, best, in the ice box, for 24 hours before filtration. The precipitate will now be found to be either a coarsely granular material or a brittle homogeneous mass on the bottom and sides of the beaker, depending on the purity of the preparation. The supernatant fluid is decanted or filtered off, the precipitate washed once or twice with cold water, and ground in a mortar with distilled water to break up the lumps. The mercury is removed by adding hot saturated barium sulfide solution in slight excess, and after stirring for a time the barium is largely removed by acidifying with sulfuric acid. The precipitated mercuric sulfide and barium sulfate are filtered off, and washed by grinding again in a mortar two or three times with more water, adding each time a small amount of barium sulfide and sulfuric acid to insure the complete decomposition of organic mercury derivatives. The combined filtrates are freed from barium or sulfuric acid, and are evaporated to dryness, *in vacuo*, to remove the excess of hydrochloric acid which is formed. The residual chlorides are taken up in water and treated with fresh silver oxide suspensions until the reaction becomes slightly alkaline; the silver chloride is filtered off and the filtrate freed from silver with  $\text{H}_2\text{S}$ . After removing the silver sulfide, the filtrate is evaporated to crystallization, *in vacuo*, and then heated on a boiling water bath to bring the crystalline material into solution. Finally, 3 or 4 volumes of boiling 95 per cent alcohol are added, and, upon standing, the sulfur compound will separate as shining crystals.

and may be filtered off, washed with alcohol, and dried; the mother liquors are concentrated further and a second group of crystals is removed in the same way. The yield of crystalline material varies from 1.5 to 2.5 gm., for each pound of casein, and will be found to be of varying degrees of purity, depending on factors which it has not yet been found possible to define or control. A sulfur determination will indicate the degree of purity, the theoretical being 21.5 per cent, and the crystals should be from 75 to 90 per cent pure. They may be rendered completely pure by carrying out a second mercuric chloride precipitation, exactly as described above, starting with a hot 10 per cent solution of the crystalline material in water, and adding about eight to ten times the weight of mercuric chloride. There is, however, an appreciable loss on reprecipitation, as well as in the first precipitation, and it has not proved possible, so far, to recover, at all quantitatively, the whole of the sulfur compound which is present in the extracts.

The resulting crystals are white and not unlike leucine or phenylalanine in appearance. Under the microscope they are found to be made up of hexagonal plates, often massed together. They are easily soluble in cold water, although when first added, they are moistened by it with some difficulty.

Combustions carried out by the Dennstedt method, permitting simultaneous determination of sulfur, gave the following results.

0.2041 gm.: 0.1384 gm. H<sub>2</sub>O, 0.3013 gm. CO<sub>2</sub>, and 0.3229 gm. BaSO<sub>4</sub>.  
0.2060 " : 0.1382 " 0.3061 " " " 0.3259 " "

Nitrogen determinations by the micro Kjeldahl method resulted as follows:

0.02158 gm. neutralized 7.34 and 7.24 cc. 0.02 N H<sub>2</sub>SO<sub>4</sub>.

C<sub>6</sub>H<sub>11</sub>SNO<sub>2</sub>. Calculated. C 40.24; H 7.43; N 9.39; S 21.50.

Found. " 40.27, 40.43; H 7.59, 7.50; N 9.53, 9.40;  
S 21.73, 21.72.

Molecular weight determinations by the ebullioscopic method, using Menzies, apparatus, with water as a solvent, gave the following results.

#### Barometric reading, corrected, 760 mm.

Weight of compound. gm.	Volume of water. cc.	Differential thermometer reading. mm.	Conversion factor.	Rise. °C.	Molecular weight.
0.3345	29.3	16.9*	0.002597	0.04389	140.5
0.4760	29.3	22.8	0.002597	0.05920	148.2
0.6834	28.5	37.7	0.002595	0.09005	143.8
Average molecular weight found .....				144.2	
Calculated " " .....				149.17	

Heated in an open capillary, the crystals begin to turn brown and shrink at 278°, and melt with decomposition quite sharply at 283°. In a sealed capillary, slight browning and shrinking of the material occurs at 274°, and it melts with decomposition at 280–281°. The readings are uncorrected.

0.4439 gm. of the compound in 16 cc. of water in a 2 dm. tube rotated the plane of polarized light  $-0.4^{\circ}$ .

$$[\alpha]_D^{20} = -7.2^{\circ}$$

It is possible that the compound is partially racemized by the extraction with hot barium hydroxide solution.

#### *Preparation of the Naphthol Isocyanate Derivative.*

0.75 gm. of pure sulfur compound was dissolved in 30 cc. of water containing 5 cc. of N KOH, and 1 cc. of  $\alpha$ -naphthol isocyanate was added. The mixture was shaken for about 5 minutes and allowed to stand for about 1 hour with occasional shaking. It was then filtered by suction and the filtrate acidified with hydrochloric acid. A heavy curdy precipitate separates which was filtered by suction, washed with cold water, and dissolved in about 50 cc. of boiling alcohol. Boiling water was then added until crystallization commenced (2 to 3 volumes), and on cooling a heavy crop of short needles separated. After filtering and washing with cold water, the crystals were dried *in vacuo* over  $H_2SO_4$  at room temperature. The yield was 1.0 gm. of material which lost no weight on further drying at 110° in an air oven.

The crystals were moderately soluble in cold acetone and 95 per cent alcohol, more so on boiling. They were not appreciably soluble in cold or hot water, benzene, chloroform, or ether.

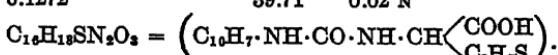
Melting point (uncorrected) 186°.

0.2056 gm.: 0.1488 gm.  $BaSO_4$ .

0.2004 " : 0.1436 " "

0.0463 " neutralized 14.68 cc. 0.02 N NaOH (Kjeldahl).

0.1272 " " 39.71 " 0.02 N "



Calculated. N 8.83, S 10.08.

Found. " 8.89, 8.75; S 9.94, 9.84.

*Composition of the Mercuric Chloride Precipitate.*

The compound produced by mercuric chloride is fairly soluble in boiling water, but separates on cooling. Prepared from a fairly pure specimen of the amino-acid, it separates from the hot solution as a sticky, oily material on the sides and bottom of the beaker, which becomes brittle as the solution cools. From a more dilute solution, it separates as a coarse, granular material, which under the microscope is made up of minute spherules. It has not proved possible to prepare it in crystalline form. The composition appears to be highly complex, corresponding, roughly, in the only specimen analyzed, to the formula  $(C_5H_{11}SNO_2)_2Hg_5Cl_8$ .

0.1058 gm.: 0.0799 gm. AgCl.

0.1223 " : 0.0878 " HgS.

1.0125 " : 0.3077 " BaSO<sub>4</sub>.

$(C_5H_{11}SNO_2)_2Hg_5Cl_8$ . Calculated. Cl 17.9, S 4.05, Hg 63.3.  
Found. " 18.7, " 4.18, " 61.9.

*Preparation of Copper Salt.*

From the purified sulfur compound, a crystalline copper salt can be easily prepared by treating the hot solution with either copper acetate solution or copper hydroxide or carbonate. The copper salt separates quickly as minute hexagonal plates, light blue in color, which are almost insoluble in cold water, and only moderately soluble in boiling water. This salt is not suitable for purification of the amino-acid, however, since the latter, in the presence of phenylalanine, yields mixed crystals of the copper salts of both amino-acids.

Some of the copper salt prepared as described, using copper acetate, was dried for analysis at 110°.

0.1759 gm.: 0.2238 gm. BaSO<sub>4</sub> and 0.0466 gm. CuS.

$(C_5H_{11}SNO_2)_2Cu$ . Calculated. S 17.82, Cu 17.66.  
Found. " 17.44, " 17.61.

*Preparation of the Compound by Alkali Hydrolysis.*

10 lbs. of casein were hydrolyzed by heating with 42 liters of 18 per cent NaOH in a stone jar under slight pressure (varying from 1 to 5 lbs. per sq. in.) in an autoclave for 14 hours. It was then cooled and neutralized with H<sub>2</sub>SO<sub>4</sub>, and after standing over night the tyrosine which had separated

was filtered off, and the filtrate precipitated by 10 lbs. of mercuric sulfate as already described, except that after the addition of the mercuric sulfate reagent, the mixture was not again neutralized, but precipitation was allowed to take place in the acid condition. It may be noted here again that under these conditions the yield is approximately half that obtained from a neutral solution, but the precipitate is less bulky and filters more easily. The precipitate, after thorough washing, was extracted ten times with 10 liters of 1 per cent  $\text{Ba}(\text{OH})_2$  solution in the cold. (The use of hot  $\text{Ba}(\text{OH})_2$  of 2 per cent concentration has been found to give a much quicker and more complete extraction.) After concentrating, and removing glutamic acid, etc., by a baryta-alcohol precipitation, which has since been found unnecessary, the material was crystallized and later purified by two precipitations with mercuric chloride as already described. The yield was about 3 gm. of pure material. The gross and microscopic appearance is practically the same as that of the material prepared by  $\text{H}_2\text{SO}_4$  hydrolysis, except that the platelets are somewhat larger and more compact.

Combustions by the Dennstedt method, and micro Kjeldahl determinations of nitrogen resulted as follows:

0.2024 gm. :	0.2984 gm. $\text{CO}_2$ ,	0.1352 gm. $\text{H}_2\text{O}$ , and	0.3175 gm. $\text{BaSO}_4$ .
0.1995 " :	0.2960 "	0.1344 "	0.3153 " "
0.02140 "	neutralized 7.14 and 7.19 cc.	0.02 N $\text{H}_2\text{SO}_4$ .	
0.01002 " :	(Van Slyke)	1.641 and 1.607 cc. N at 19° and 767 mm.	
$\text{C}_{8}\text{H}_{11}\text{SNO}_2$ . Calculated.	C 40.24, H 7.43, N 9.39, S 21.50.		
Found.	" 40.18, 40.46; H 7.47, 7.54; N (total) 9.35,		
	9.41 and N (amino) 9.45, 9.26; S 21.54, 21.70.		

Heated in an open capillary the substance melts with decomposition at 265–266°.

In a sealed capillary it melts and decomposes at 262–264°.

0.3133 gm. dissolved in 16 cc. water in a 2 dm. tube did not rotate the plane of polarized light.

#### *Preparation from Egg Albumin.*

10 lbs. of dry commercial egg albumin were hydrolyzed with 30 liters of water and 30 lbs.  $\text{H}_2\text{SO}_4$  by heating in a stone jar, placed in an autoclave without pressure, for 20 hours. The resulting solution was neutralized with  $\text{Na}_2\text{CO}_3$  and precipitated with 10 lbs. mercuric sulfate, and the mixture neutralized with  $\text{NaOH}$ . The resulting precipitate, after washing, was extracted seven times with about 14 liters of cold  $\text{Ba}(\text{OH})_2$  solution, about 1.3 per cent. The extracts after concentrating and precipitating with baryta and alcohol were concentrated and the resulting mixture of amino-acids was recrystallized. The ethyl ester hydrochlorides were prepared by suspending the dry crystals weighing 12.9 gm. in absolute alcohol and saturating with dry  $\text{HCl}$  gas in the usual way. The esters were liberated after distillation of the alcohol, by anhydrous  $\text{Ba}(\text{OH})_2$  added to an ether

suspension cooled by ice. The ethereal solution was filtered off, the ether distilled *in vacuo* at room temperature, and the esters were distilled from an oil bath in a vacuum produced by a Geryk pump. A small amount of ester passed over before the bath reached 100°, which has not been identified. More rapid distillation began with the bath at a temperature of 120°, and the vapor at 80–90°. At 90° the distillation was interrupted, the receiver changed, and a fraction collected with the bath between 120 and 160°, and the vapor between 92 and 116°. About half the material had not passed over, and the distillation was carried no further.

The first fraction of distillate (vapor up to 90°) contained only a small amount of sulfur and was not examined further. The second fraction (vapor 92–116°) contained much sulfur, as did the distillation residue. The latter on cooling set to a mass of whitish semicrystalline material. This was insoluble in water and moderately soluble in hot ethyl acetate from which it crystallized on cooling. The resulting crystals apparently did not represent a pure compound, as analysis showed them to have the following composition.

Diketopiperazine of  $C_8H_{11}SNO_2$ ,  $C_{10}H_{18}S_2N_2O_2$ .  
Calculated. C 46.10, H 6.20, N 10.76, S 24.65.

Found. C 51.46, H 6.87, N 10.10, S 17.90.

The esters distilling between 92 and 116°, weighing 6.8 gm. were poured into about 20 cc. of water, in which the greater part appeared to dissolve. This was extracted with an equal volume of ether. The aqueous phase was run off and the ether washed twice with 1 to 2 cc. of water, the washings were added to the first aqueous solution. The combined aqueous solution was now washed twice with 3 to 4 cc. of ether to remove as much remaining phenylalanine as possible. The solution was heated on a water bath under an air condenser for 4 hours until the alkaline reaction had disappeared, evaporated with the addition of a little alcohol to facilitate crystallization, and recrystallized once. The yield was only 0.35 gm. The ether solution contained the greater part of the sulfur compound ester mixed with the phenylalanine ester.

Analysis of the 0.35 gm. lot gave the following results.

0.2002 gm.: 0.3019 gm.  $CO_2$ , 0.1314 gm.  $H_2O$ , and 0.3039 gm.  $BaSO_4$ .  
0.0199 " neutralized 6.56 cc.  $H_2SO_4$ .  
0.0202 " " 6.71 " " The  $H_2SO_4$  was approximately 0.02 N, of which 1.0 cc. was equivalent to 0.2788 mg. N.  
Found. C 41.44; H 7.34; N 9.19, 9.26; S 20.85.

It was from this preparation that the formula  $C_5H_{11}SNO_2$  was first deduced for the compound. It agrees quite well with that calculated for a mixture containing 97 per cent  $C_5H_{11}SNO_2$  and 3 per cent phenylalanine.

*Preparation from Other Proteins.*

No attempt has been made to compare, by the method given above in detail, the yield of sulfur compound obtained from other sources than casein. As already stated, it has been prepared from egg albumin, the yield being roughly the same as from casein. It has also been prepared in an impure form from edestin and wool, the yield being about the same as from casein, and from gelatin, although from the latter protein very little resulted. In the case of wool, which contains most of its sulfur in the form of cystine, it seemed desirable to find out if possible just how much of the sulfur could be accounted for as cystine. A small sample of the same lot of wool which had been used for preparing the new amino-acid was carefully washed with water, alcohol, and ether and dried, and the total sulfur determined by burning 1.242 gm. in the Dennstedt furnace.

0.3030 gm.  $BaSO_4$ , corresponding to 3.35 per cent sulfur was obtained.

Through the courtesy of Dr. J. M. Looney of the Biochemical Laboratory, Harvard Medical School, the cystine was determined on part of the same washed wool, and found to be 9.1 per cent, corresponding to 2.43 per cent of sulfur. Roughly, 0.9 per cent of sulfur, therefore, remained not accounted for, a part of which may have been in the form of  $SO_4$ .

*Evidence as to the Existence of the Compound in the Protein Molecule.*

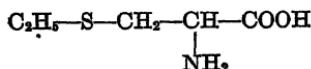
It has been known for some time that the sulfur which is in the protein molecule is not entirely in the form of cystine. Osborne (2) has reviewed the matter thoroughly and has made a careful quantitative study of the proportion of lead blackening to firmly bound sulfur. Whether the amino-acid described in the present paper can be regarded as accounting for a part of the firmly bound sulfur cannot be regarded as definitely established at present, although the evidence furnishes strong indication for so believing.

If it has been produced through a secondary reaction following hydrolysis, the sulfur in the compound must result either from the reagents used, or from some other sulfur-containing nucleus in the protein. In regard to the latter possibility, it has at least been shown that wool, which contains much cystine, gives no larger yield of the compound than does casein, in which cystine, if present at all, is in minimal amounts. As far as sulfur from the reagents is concerned, the evidence is more direct. Sulfuric acid can be definitely ruled out since sodium hydroxide can be used equally well for hydrolysis, and while the precipitation is carried out in the presence of neutral sulfate, it is highly improbable that a compound of the type described could result from such a source. The compound has also been prepared from "aminoids," a commercial protein hydrolysate prepared by enzymes. Sulfur from  $H_2S$  which may be used in the removal of mercury in place of  $BaS$ , has been eliminated in one experiment by the use of hydrogen selenide, which is just as effective in separating the mercury, and while the resulting crystals were not freed from phenylalanine, they corresponded in all their properties to similar mixtures obtained in the usual way.

#### *Structure of the Compound.*

The definite structure of the amino-acid has not yet been determined. The type of sulfur linkage is particularly puzzling. From the proportion of hydrogen to carbon, a ring of some sort is less probable than an aliphatic structure. A hydrogen replaceable by metals is present, since the copper salt corresponding to the formula  $(C_5H_{10}SNO_2)_2Cu$  can be readily formed. The replaceable hydrogen is probably attached to a COOH group, and not an SOOH group, since heating in a dry tube leads to an evolution of  $CO_2$ . Moreover, at the same time a sulfur-containing complex is split off, having an odor suggesting boiled cabbage, which gives a strong reaction with sodium nitroprusside in alkaline solution. The nitrogen is present in the  $NH_2$  form, probably in the  $\alpha$  position, since it is given off quantitatively in the Van Slyke amino nitrogen apparatus in 3 minutes. An asymmetric carbon atom is indicated by the optical rotation of the product of acid hydrolysis. Sufficient material has not been available to carry out satisfactory oxidation experiments.

It was suggested to me by Dr. Stewart of the Biochemical Laboratory at Cambridge University, that the compound might be ethyl cysteine, a thio ether, having the structure



This compound was prepared by Brenzinger (4) and by Neuberg and Mayer (5) during the study of the structure of cystine. Its properties as described by them, correspond in some, but not in all, points with the compound here described. Preparation of the ethyl cysteine by the method of Brenzinger, from cystine, proved a simple matter, and the resulting crystals were identical in gross appearance and crystal form with the new compound, but the melting point was definitely lower. The composition of the ethyl cysteine was found to be correct by complete analysis. The chemical properties of the two substances, however, are quite different, since ethyl cysteine, on boiling with even fairly weak NaOH solution (2 to 3 per cent) is broken up with an evolution of ethyl mercaptan and ammonia, while the new compound treated in the same way is apparently quite stable, and, therefore, obviously has a different structure.

The writer hopes to be able to carry out further work on the structure and possible synthesis in the near future.

#### CONCLUSIONS.

A new amino-acid, which apparently has the formula  $\text{C}_5\text{H}_{11}\text{SNO}_2$ , has been isolated from the sulfuric acid hydrolysis products of several proteins, and from casein also after hydrolysis with sodium hydroxide. The yield from casein varies from 0.2 to 0.4 per cent, and is probably not quantitative.

While the writer wishes to be extremely conservative in presenting this compound as a primary cleavage product of protein, there is a certain amount of evidence to indicate that it is not a secondary decomposition product, but is present as such in the protein, and will account for at least a part of the non-lead blackening, or firmly bound sulfur.

The structure has not yet been determined.

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## STUDIES IN THE PHYSIOLOGY OF MUSCULAR EXERCISE.

### IV. BLOOD REACTION AND BREATHING.

By DAVID P. BARR.

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The researches of Winterstein (1), Hasselbalch (2), Haldane (3), and others led to the conclusion, widely accepted, that the activity of the respiratory center is dependent upon the reaction of the blood. According to Campbell, Douglas, Haldane, and Hobson (4), the regulation by blood reaction is so sensitive that a diminution in alkalinity of only pH 0.012 is sufficient to double the respiration while a similar increase in alkalinity may produce apnea. An excellent opportunity of determining the validity of this statement and the true relationship between reaction of the blood and respiration is furnished by experiments with muscular exercise. It is common knowledge that great changes in pulmonary ventilation occur as a result of exertion. In the preceding papers of this series it has been shown by some forty experiments that changes in blood reaction are also great. It remains to be seen whether a quantitative relationship between the two phenomena can be demonstrated.

The unexpectedly large changes in blood reaction which were found in our experiments 3 or more minutes after the cessation of work (5), made us suspect at an early stage in the research that the quantitative relationship, stated by Campbell, Douglas, Haldane, and Hobson could not be strictly applied to muscular exercise. Since the volume of respiration was not measured in any of the earlier experiments, however, this idea was only an impression which we later endeavored to investigate. In many of our experiments, the work was standardized to approximately 3,500

kilogrammeters in  $3\frac{1}{2}$  minutes. It was believed that a general idea of the relationship between reaction and respiration could be obtained by the measurement of minute volume of breathing in normal individuals during and after the performance of the standard amount of work. A comparison of these changes in breathing could then be made with the variations in blood reaction which had been noted under similar conditions in other experiments.

A continuous record of the volume of respiration was obtained in four normal men before, during, and after exercise corresponding to approximately 3,500 kilogrammeters in  $3\frac{1}{2}$  minutes. Upon three of these subjects (D.P.B., H.E.H., and K.G.H.) a study of blood reaction accompanying exercise had previously been made (5). The method of measuring respiratory volume was as follows. A closely fitting French Tissot gas mask was adjusted to the face of the subject. It was held firmly in position over the malar bones with padding and straps and carefully tested for leaks. The mask was connected with two large rubber tubes (inside diameter 1 inch) one of which conducted air for inspiration, the other expired air to either of two 80 liter Tissot spirometers. Inspired and expired air were separated by rubber flutter (Saad) valves of the type used in gas masks. The volume of respiration was read off on the spirometer every half minute during and following exercise. The observed volume was corrected to 760 mm. and  $37.5^{\circ}\text{C}.$ , wet.

Although the total volume differed the general character of the respiratory response did not vary in the four subjects. In all of them, the maximum volume was found during the last minute of exercise. The volume fell rapidly for about 5 minutes and gradually for 15 or more after the work was stopped. It was still several liters greater than the original resting volume 15 minutes after the exertion. The respiratory response of H.E.H. is given in Fig. 1 where the minute volume is charted in relation to the time during or after exercise. The curve differs in no essential from those of the other normal individuals and may be taken as the usual reaction of a normal man to the standard amount of work.

Upon H.E.H. three experiments were done in which the reaction of blood was determined before, during, and at varying times after the performance of approximately 4,000 kilogrammeters in  $3\frac{1}{2}$  minutes. The protocols and original report of those experiments may be found in Paper III of this series (6). In Table I, a com-

parison has been made between the reaction of blood found in these three experiments with the volume of breathing which occurred at similar times in the observation just reported. It will be seen that the change in reaction of blood was less during exercise when the breathing was greatest than it was later at a time

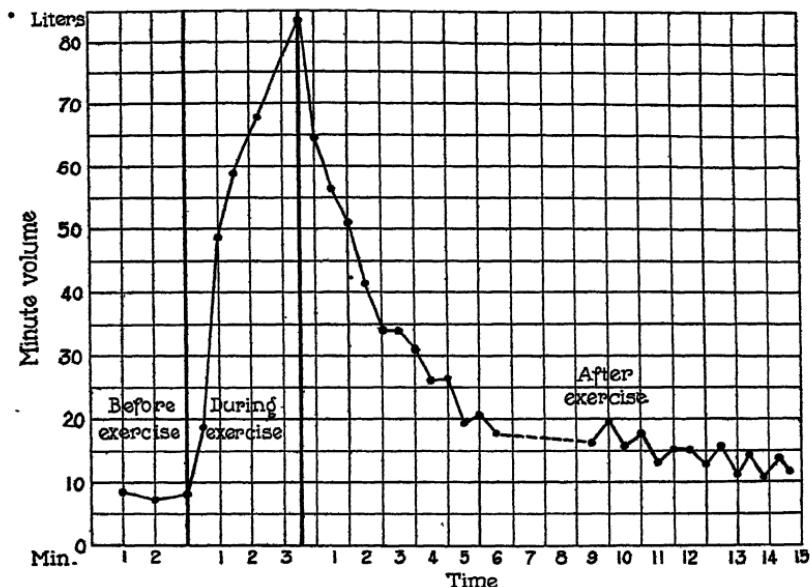


FIG. 1. Respiratory response of H. E. H. to 3,800 kilogrammeters of work in 3½ minutes.

TABLE I.  
*General Relation of Respiration to Reaction in H. E. H. during and following Exercise.*

Time relation to exercise.	Minute volume May 23.	pH of arterial blood.		
		Apr. 14	Apr. 5	Apr. 21
	Liters			
Before.....	8.0	7.35	7.30	
During last minute.....	83.5	7.27		
1 min. after.....	56.0		7.16	
3 " "	34.0		7.15	7.19
15 " "	11.0			7.23

when the pulmonary ventilation was rapidly diminishing. Moreover, the alkalinity was slightly greater 1 minute after exercise than after 3 minutes, although the volume of breathing had further diminished rapidly during the interval. The comparison indicates that the reaction of blood was changed much more after exercise than could be expected from the change in breathing if the calculation of Campbell, Douglas, Haldane, and Hobson were employed. For example, the reaction in the experiment of H.E.H. on April 5 was pH 7.30 before exercise and 7.15 after the work had ceased, a difference of 0.15. A change in pH of 0.15 should, according to the calculation, be accompanied by a pulmonary ventilation  $\frac{0.15}{0.012}$  or some twelve and a half times the normal, about 100 liters. We found at this time a minute volume of only 29 liters. The large change in reaction which was observed in H.E.H. 3 minutes after exercise was no greater than that seen in many experiments reported by Barr, Himwich, and Green (5). In all of these, the calculation would indicate a volume of breathing much larger than could have occurred.

It is not likely that the general character of the respiratory response of H.E.H. varied considerably on different days, nor is there any reason to believe that his response differed significantly from that of other normal individuals doing the same amount of work. However, since conditions were not identical in the different experiments a comparison of the breathing on 1 day with the reaction of the blood on another cannot be entirely convincing. Furthermore, this sort of comparison does not allow us to make accurate quantitative application of the calculation of Campbell, Douglas, Haldane, and Hobson.

Several other experiments were performed in which the volume of breathing was measured during the time when the arterial blood was being drawn. In these, a slightly different technique was employed for the determination of minute volume. The breathing was not measured continuously, but only during the periods in which the blood was taken. Instead of the gas mask, a mouthpiece and nose-clip were used. The gut valves of Pearce (7), which offer almost no resistance to respiration, were substituted for the Saad valves and were attached to a wide caliber T-tube. The total instrumental dead space of the apparatus was approximately 35 cc.

The results of five experiments in which the respiration and reaction of arterial blood were studied simultaneously are given in

TABLE II.  
*Relation of pH to Minute Volume of Breathing.*

Subject.	Date.	Time relation to exercise.	pH	Minute volume ob- served.	Minute volume calcu- lated.	Remarks.
D. P. B.	Oct. 17	Before.	7.26	10.0	liters	Work = 6,162 kg. m. in 5 min. 22 sec. Blood taken before and 7 min. after exercise.
		During.		81.5		
		After.	7.07	26.2		
D. P. B.	Oct. 24	Before.	7.31	10.8	liters	Work = 3,504 kg. m. in 3 min. 20 sec. Blood taken before and 15 min. after exercise.
		During.		59.0		
		After.	7.23	14.0		
M. F.	Oct. 19	Before.	7.28	18.2	liters	Work = 4,074 kg. m. in 4 min. 30 sec. Blood taken before, during last min. of exercise, and 5 min. afterwards.
		During.	7.13	60.0		
		After.	7.09	31.7		
H. E. H.	Aug. 25	Before.	7.28	7.9	liters	Work = 3,545 kg. m. in 3 min. 20 sec. Blood taken before, during last min. of exercise, and 4 min. afterwards.
		During.	7.20	51.0		
		After.	7.21	14.6		
H. E. H.	Oct. 27	Before.	7.32	7.0	liters	Work = 3,408 kg. m. in 3 min. 30 sec. Blood taken before and during 2nd min. of exercise.
		During.	7.25	49.5		

Table II. The protocols of the blood studies may be found in Paper III of this series (6). In the table, there is included a comparison between the observed difference in minute volume and

that which would have been calculated from the change in reaction by the rule of Campbell, Douglas, Haldane, and Hobson. In the experiment on M.F. the respiration was increased before the exercise started. The subject was comparatively inexperienced and dreaded the arterial puncture which seemed to be rather painful. Although it is quite evident that he was overventilating, this increased breathing should reflect itself on the blood reaction and so should introduce no considerable error in comparison of conditions before and after exercise.

A study of Table II reveals additional evidence that the rule of Campbell, Douglas, Haldane, and Hobson cannot be applied to the conditions following muscular exercise. The respiratory center is much less sensitive to changes in reaction than they supposed. After exercise in every case, the calculated respiration was much greater than that which was actually observed. During exercise, in two instances, the discrepancy between observed and calculated values was not great (H.E.H., August 25, 1922 and October 27, 1922). In a third (M.F., October 19, 1922), however, the calculated respiration was enormously greater.

No constant relationship between reaction and respiration can be demonstrated from these experiments. After exercise when the minute volume was rapidly decreasing the acidosis of the arterial blood remained essentially the same or even increased. Thus, in the observation on H.E.H., August 25, 1922, the pH was 7.20 with a minute volume of 51.0 liters and only 7.21 with a volume of 14.6 liters 3 minutes later. In M.F., October 19, 1922, with  $4\frac{1}{2}$  minutes work the pH was 7.13 with a ventilation of 60 liters during the last minute of exercise and 7.09 with a minute volume of 32 liters 5 minutes after the work was complete. As may be seen by reference to the protocols of Paper III, this progressive decrease in alkalinity after exercise was found in other experiments in which the respiration was not measured simultaneously.

At the time our investigation of muscular exercise was commenced, we shared the common belief that blood reaction furnishes the normal stimulus to respiration. The results which have been described came as a distinct surprise. As the absence of any relationship between breathing and blood reaction became more and more apparent, we made a review of the literature first to ascertain the experimental basis for the widespread belief in pH

control and second to find out whether other observers had obtained results similar to ours.

In 1905, Haldane and Priestley (8) demonstrated that the volume of respiration varies with the tension of CO<sub>2</sub> in the alveolar air under a wide range of conditions. The breathing of mixtures rich in CO<sub>2</sub> raised the tension of CO<sub>2</sub> in the alveolar air and increased the volume of respiration. Forced breathing of air, on the other hand, diminished the CO<sub>2</sub> in the alveoli and caused apnea. From the results of a series of experiments Campbell, Douglas, and Hobson (9) showed that an increase of approximately 0.2 per cent in the alveolar CO<sub>2</sub> doubled the alveolar ventilation. A reduction of the same amount produced apnea. This was found to apply to moderate amounts of exercise. It seemed possible by a study of the tension of CO<sub>2</sub> in the alveolar air to predict the degree of increase in the volume of respiration. When more strenuous work was done, however, the rule no longer applied. Douglas and Haldane (10) found in walking experiments that the alveolar CO<sub>2</sub> was lower with very rapid walking than it was when the walking was done at a more moderate rate. The volume of breathing was, of course, greater with the more strenuous exertion. Furthermore, after the very strenuous exercise of running up and down stairs, Douglas and Haldane (11) found that the alveolar CO<sub>2</sub> remained low for about 1 hour. Under these conditions, the breathing was moderately increased. According to the hypothesis of CO<sub>2</sub> control of respiration, an apnea should have been expected. With the evidence of these later experiments, it was no longer possible to maintain that CO<sub>2</sub> acted specifically as an excitant to the respiratory center during and after strenuous exercise.

As a result of perfusion experiments on new-born rabbits, Winterstein (1) in 1911 formulated the hypothesis that the reaction of blood is the true respiratory hormone. A year later, Hasselbalch and Lundsgaard (12) showed a definite relationship between CO<sub>2</sub> pressure and the reaction of blood. In a study of the effect of diet, Hasselbalch (2) showed that alveolar CO<sub>2</sub> tension might be altered considerably, but in such a way that the reaction of the blood remained practically normal. From these experiments and others, Hasselbalch came to the conclusion that the minute volume of respiration is so adjusted as to maintain the reaction of the blood constant and that the CO<sub>2</sub> excites the respiratory center only by

its action as an acid in solution. This idea was accepted by Haldane and his associates. In 1914, Christiansen, Douglas, and Haldane (13) demonstrated a great reduction in the bicarbonate of the blood following exercise, which they attributed to the accumulation of lactic acid. They concluded that under these circumstances the respiratory center responds to the combined effects of carbonic and lactic acids upon the reaction of the blood and that the changes in reaction are responsible for the variations in breathing. Hasselbalch and Lundsgaard (12) had found that a rise of 0.2 per cent in  $\text{CO}_2$  tension corresponds to a fall of 0.012 in pH. Applying this knowledge to the results already obtained on alveolar  $\text{CO}_2$  tension, Campbell, Douglas, Haldane, and Hobson (4) stated the method for predicting respiratory changes in terms of blood reaction which has been discussed in the preceding pages.

With forced breathing or with the breathing of  $\text{CO}_2$ -rich mixtures it was difficult to prove this assumption because the theoretical changes in pH often fell within the limits of error of any method then or now available. During exercise, however, in which the volume of respiration is enormously increased, the changes in pH demanded by the theory should have been easily measurable and the correctness of the assumption should have been susceptible to proof. Few studies were made on the reaction of the blood after exercise. Changes in pH after exercise were tested by Barcroft (14) using as an index the effect of exercise upon the dissociation of oxyhemoglobin. In a climb of 1,000 feet up Garlingford mountain, he found a change in pH of 0.20 in blood drawn from his finger immediately after the ascent. According to the hypothesis of Campbell, Douglas, Haldane, and Hobson (4) this change in pH should have caused a rise of something over 100 liters in his ventilation. It is clear, however, from Barcroft's description of his climb that he experienced no respiratory distress. The rate chosen was such that "respiration could comfortably be performed through the nose."

Parsons, Parsons, and Barcroft (15), using direct electrometric methods, found on the finger blood of Barcroft a change in pH of 0.08 during the last moments of exercise in which the volume of breathing had increased from 8 to 29 liters. This indicated a sensitivity of the respiratory center about one-half of that which would be deduced by the calculation of Campbell, Douglas,

Haldane, and Hobson. Parsons obtained a similar result in comparing the arterial blood of Barcroft at rest and while working under conditions of low oxygen pressure.

In the experiments of Douglas and Haldane (11) in which Douglas ran up and down stairs, it was found that 10 minutes after exercise, his blood had lost about 40 per cent of its bicarbonate and that the alveolar tension was diminished by about 20 per cent. Hasselbalch (16) applied the data to the indirect method of estimating reaction from the carbonic acid-bicarbonate ratio and calculated that the pH of Douglas's arterial blood must have fallen by 0.12. This would be sufficient according to the theory of Campbell, Douglas, Haldane, and Hobson to increase the volume of respiration about ten times its resting value. According to the evidence, however, there was at this time no visible hyperpnea.

In addition to the experiments with muscular exercise, there has accumulated in the literature some evidence which casts doubt on the conception that blood reaction is always the controlling factor in breathing. Scott (17), using a colorimetric method of pH determination, found that with the proper injection of alkali, the arterial blood might be made as alkaline as pH 7.8 without changing the character of the normal respiration. The minute volume might be the same within a few cubic centimeters whether the pH of the blood was 7.4 or 7.8.

It was found by Haldane, Kellas, and Kennaway (18) that the hyperpnea which occurs at altitudes may produce an alkalosis and that the hyperpnea may continue in the presence of the alkalosis. Similar alkalosis was found by Haggard and Henderson (19). Haldane, Kellas, and Kennaway believed that the hyperpnea resulted from an increased sensitivity of the respiratory center due to anoxemia. Haggard and Henderson, not content with this explanation, suggested that in the presence of low oxygen tension, a substance was evolved which acted specifically as a respiratory stimulant, produced the original hyperpnea, and caused its continuance even in the presence of an alkalosis. Haggard and Henderson (20) have found a similar increase in breathing in the presence of an alkalosis after carbon monoxide poisoning.

With ether anesthesia in dogs, Van Slyke, Austin, and Cullen (21) obtained great changes in the reaction of blood. In one of

their experiments (No. 5), the volume of breathing was normal at a time during the anesthesia when the pH of the blood had been reduced by 0.16. Later in the experiment, when the reaction was returning to normal, the minute volume of breathing was more than doubled. Similar discrepancies were found in another of their experiments (No. 6) in which the ventilation and reaction were measured simultaneously.

The evidence which has accumulated in the literature indicates that the reaction of the blood does not furnish as specific and delicate a control of respiration as was originally supposed. Different methods of pH determination, electrometric and indirect, agree in showing the respiratory center comparatively insensitive to changes in blood reaction after exercise. The experiments of Barcroft and Parsons are in agreement with our observations. The calculations of Hasselbalch on the results of Douglas and Haldane indicate the same lack of sensitivity. Other experiments in the literature demonstrate that in a variety of conditions, great variations in blood reaction may occur without corresponding changes in the volume of breathing. In fact, the two phenomena in many instances seem to have no relation to each other. Thus, Pearce produced a marked alkalosis without change in pulmonary ventilation. Haggard and Henderson found an increased breathing in the presence of an alkalosis both at altitudes and in carbon monoxide poisoning. Van Slyke, Austin, and Cullen in ether anesthesia observed a severe acidosis without increase in the pulmonary ventilation. In our experiments, there was an increasing acidosis at a time when the volume of breathing was rapidly diminishing.

Haldane abandoned his original hypothesis of specific control of respiration by CO<sub>2</sub> tension when it failed to explain conditions during and following severe muscular exercise. The experiments which are here reported together with the evidence in the literature indicate that the hypothesis of respiratory control by blood reaction answers the requirements no better and is equally untenable.

It is, of course, possible that blood reaction does furnish the normal stimulus to breathing and that the lack of apparent relationship between reaction and respiration in our experiments and in those of others is due to changes in the sensitivity of the respiratory center under varying conditions. If this be true, it is

apparent that there must be many influences which modify or even obliterate the effect of the normal stimulus. Of these, ether anesthesia, exercise, properly controlled alkali injections, low oxygen tension, and carbon monoxide poisoning are examples. With so many different conditions changing the sensitivity of the respiratory center, however, blood reaction can no longer be regarded as the determining factor in the control of breathing.

#### SUMMARY AND CONCLUSIONS.

1. The reaction of arterial blood and the minute volume of respiration have been studied simultaneously before, during, and after 3½ minutes of vigorous muscular exercise.
2. It is found that following exercise, the respiratory center is much less sensitive to changes in reaction of arterial blood than has been formerly supposed.
3. No constant relationship between blood reaction and breathing can be demonstrated. Following exercise, there may be an increasing acidosis in the arterial blood at a time when the pulmonary ventilation is rapidly diminishing.
4. A review of the literature reveals the fact that much evidence has accumulated against the idea that blood reaction is the determining factor in the control of breathing.

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## DETERMINATION OF AMINO NITROGEN IN COMPOUNDS REACTING SLOWLY WITH NITROUS ACID.\*

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Van Slyke (1) pointed out in an early communication describing his method for the determination of amino nitrogen, that certain groups react slowly with nitrous acid and may react completely only after a considerable time varying from 30 minutes to 8 hours at 20°. The statement was made that the purine and pyrimidine derivatives (except guanosine) react normally in from 2 to 5 hours at 20°. Data were given for determinations of cytidine,<sup>1</sup> adenosine, and guanosine although the time of reaction was not specifically stated.

While making a study of the rate of reaction of adenine nucleotide, it was found desirable to extend the observations to other compounds. The data obtained are given in detail in Tables I to IV and are summarized in the form of curves showing the rate of reaction (Figs. 1 and 2). The periods of the reactions are plotted as abscissæ and the quantities of gas liberated (calculated in terms of percentage of one reacting nitrogen atom in the molecule) are plotted as ordinates. The temperatures at which most of the reactions took place varied in different experiments from 21 to 26°. The method was used as described by Van Slyke (3), employing the micro apparatus. After mixing the solutions in the reacting chamber, they were allowed to stand the desired time, shaking only the last 1 or 2 minutes.

The corrections for the reagents were determined, using 2 cc. of water (no antifoaming material was used in any of these experi-

\* Reported at the meeting of the American Society of Biological Chemists (Wilson, D. W., *J. Biol. Chem.*, 1920, xli, p. i).

<sup>1</sup> Levene and Jacobs (2) report that cytidine reacts completely in 2 hours.

ments), and carrying out the determination as usual. The following blanks were found: 3 min., 0.09 cc.; 30 min., 0.12 cc.; 1 hr., 0.15 cc.; 2 hrs., 0.19 cc.; 3 hrs., 0.22 cc. It should be emphasized that, especially for the determinations of long duration, it is absolutely essential that the stop-cocks be well ground and thoroughly greased with heavy stop-cock grease or serious errors will arise due to slight leaks. In these experiments the stop-cocks were frequently reground with powdered emery. The stop-cock at the upper end of the gas burette was greased after every three or four determinations.

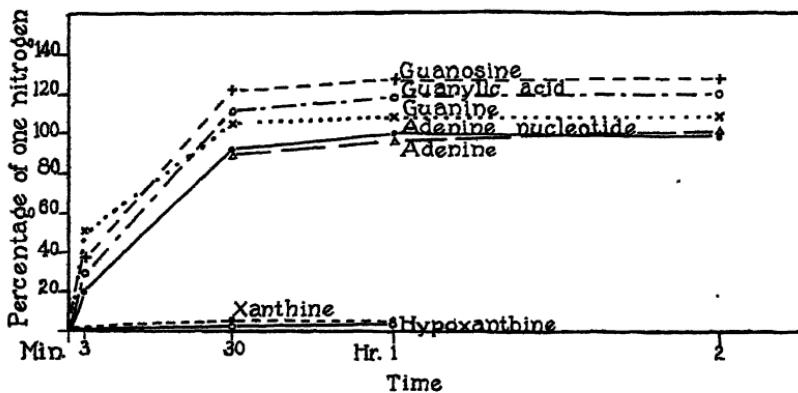


FIG. 1.

Inspection of the curves brings out several points of interest. As may be seen, guanosine yields in 30 minutes more gas than can be accounted for by assuming that one amino group has reacted. At the end of 1 hour the unabsorbed gases total 27 per cent more than the amount required for one amino group. Thereafter, there is no appreciable increase. This confirms Van Slyke's observation with the same compound, although the reaction was found to be practically complete in 1 hour instead of 2 to 5 hours. Guanylic acid behaves in the same way, yielding about 20 per cent more than the theoretical amount of gas. While guanine reacts similarly, only about 8 per cent excess was observed. There is no question about the purity of the materials.<sup>2</sup> The

<sup>2</sup> The purines and pyrimidines and derivatives of yeast nucleic acid were kindly furnished by Dr. Walter Jones.

TABLE I.

Quantity of material* and of one reacting nitrogen.	Time.	Gas volume corrected.	Temperature.	Barometer.	Weight of N found.	Percentage of one reacting nitrogen.
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## Guanine HCl.

mg.		cc.	°C.	mm. Hg	mg.	
11.13 0.698 N	3 min.	0.65	26.0	758	0.358	51
	30 "	1.31	23.0	763	0.737	106
	1 hr.	1.35	23.7	759	0.754	108
	2 hrs.	1.37	26.5	758	0.752	108

## Guanosine.

15.90 0.698 N	3 min. 30 " 1 hr. 2 hrs.	0.48 1.54 1.59 1.60	.24.5 24.5 24.0 24.7	757 757 757 757	0.266 0.854 0.884 0.886	38 122 127 127
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## Guanylic acid.

16.8 0.618 N	3 min. 30 " 1 hr. 2 hrs.	0.33 1.20 1.30 1.31	22.8 21.2 22.8 22.8	769 769 762 769	0.187 0.687 0.732 0.744	30 111 119 120
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## Adenine picrate.

19.9 0.730 N	3 min. 30 " 1 hr. 2 hrs.	0.26 1.17 1.25 1.32	24.7 22.7 24.7 24.9	761 761 761 761	0.145 0.658 0.697 0.733	20 90 96 100
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## Adenine nucleotide.

17.1 0.656 N	3 min. 30 " 1 hr. 2 hrs.	0.23 1.06 1.16 1.17	23.2 23.3 22.2 23.2	753 762 762 753	0.128 0.596 0.654 0.648	20 91 100 99
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\* In Column 1 is given the calculated amount of reacting nitrogen in the material, assuming that only one nitrogenous group reacts. This amount is used to calculate the percentages in the last column.

guanosine was crystalline. Guanine hydrochloride which had been recrystallized and dissolved in 2 per cent HCl was used in the guanine estimations. Van Slyke states that he was unable to analyze guanine because it precipitated in the reacting chamber. There is a tendency for it to precipitate, but this may be overcome if the solution is introduced slowly into the reacting chamber. Some xanthine separates out in the longer experiments. There is adequate reason to assume that the observed differences are due to actual differences in the reactions of the compounds.

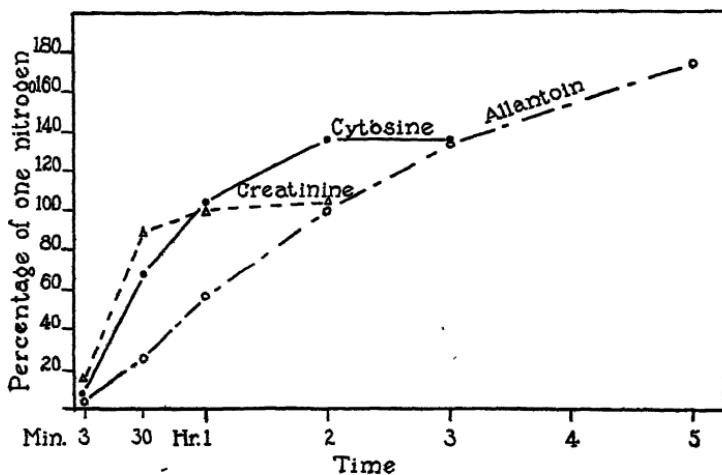


FIG. 2.

In contrast with guanine and its derivatives, adenine and adenine nucleotide yield exactly the theoretical amount of nitrogen by this method. The crystalline adenine nucleotide and adenine sulfate and picrate were used. It is interesting to note that the two curves showing the rates of reaction of adenine nucleotide and adenine practically coincide, proving that the combination of adenine in the nucleotide does not influence the rate of reaction of the amino group.

Xanthine and hypoxanthine each give off small quantities of gas not absorbed by alkaline permanganate. No gas is liberated from uric acid.

The pyrimidine, cytosine, reacts maximally with nitrous acid in 2 hours at 24°, yielding 35 per cent more than the calculated amount of nitrogen. The abnormal behavior is thus more pronounced than that of guanosine. In these experiments cytosine sulfate was used which had been recently recrystallized from diluted sulfuric acid and found to contain the theoretical percentage of nitrogen [(cytosine)<sub>2</sub> H<sub>2</sub>SO<sub>4</sub> · 2H<sub>2</sub>O]. This curiously

TABLE II.

Quantity of material and of one reacting nitrogen.	Time.	Gas volume corrected.	Temper-ature.	Barometer.	Weight of N found.	Percentage of one reacting nitrogen.
Cytosine sulfate (23.36 per cent N; theory 23.59 per cent N).						
mg.	cc.	°C.	mm. Hg	mg.	mg.	
10.1 0.794 N	3 min. 30 " 1 hr. 2 hrs. 3 "	0.11 0.98 1.51 1.95 1.96	25.0 24.5 24.5 24.5 23.7	745 745 745 751 752	0.060 0.534 0.824 1.072 1.083	8 67 104 135 136
Allantoin (Grübler).						
12.4 1.097 N  1.152 N 0.877 N	3 min. 30 " 1 hr. 2 hrs. 3 " 5 "	0.06 0.50 1.07 1.97 2.83 2.73	23.3 24.0 24.5 24.0 25.2 25.5	764 764 764 764 755 765	0.034 0.281 0.599 1.105 1.560 1.522	3 26 55 101 135 174
Creatinine.						
6.27 0.776	3 min. 30 " 1 hr. 2 hrs.	0.22 1.22 1.37 1.44	22.4 21.2 22.0 23.0	759 764 759 759	0.124 0.694 0.772 0.806	16 89 100 104

abnormal reaction of cytosine is of peculiar interest because Van Slyke reported that cytidine reacts to give exactly the theoretical quantity of gas. This has been confirmed by a single determination, using pure, analyzed cytidine nitrate which yielded 93 per cent of one nitrogen in 2 hours.

Neither thymine, uracil, nor uridine gives any nitrogen after standing with nitrous acid 1 or 2 hours.

Allantoin reacts more slowly than the amino-purines and amino-pyrimidines, but has not ceased reacting at the end of 5 hours when nearly 2 atoms of nitrogen have been accounted for.

A few other compounds have been examined. Brucine yields negative results. Substituted amino groups such as are found in betaine (trimethyl glycocoll) and sarcosine (methyl glycocoll) do not react. Guanidine and methyl guanidine both yield small quantities of gas.

TABLE III.

Substance.	Quantity used. mg.	Time.	Gas volume corrected. cc.	Temperature. °C.	Barometer. mm. Hg	Weight of N found. mg.	Percentage of one reacting nitrogen.
Cytidine nitrate.....	15.6	2 hrs.	1.20	24.3	751	0.661	93
Hypoxanthine.....	9.7	30 min.	0.05	25.7	761	0.028	2.8
	9.7	1 hr.	0.08	25.7	761	0.044	4.4
Xanthine.....	10.75	30 min.	0.09	24.0	763	0.050	5.1
	10.75	1 hr.	0.10	24.0	763	0.056	5.7
Thymine.....	14	1 "	0.0	23.0			
Uric acid.....	10.6	30 min.	0.01	22.8	774		
Uridine.....	23.2	1 hr.	0.0	23.0			
Creatine.....	16	30 min.	0.03	20.8	774		
Uracil.....	11.4	2 hrs.	0.01	24.0	765		
Brucine sulfate.....	10.20	1 hr.	0.0				
Betaine HCl.....	50	1 "	0.0				
Sarcosine.....	6.8	30 min.	0.0	22.3	762		
Triphenylguanidine....	8.5	30 "	0.0	22.5	762		

Van Slyke reports that creatine does not react with nitrous acid. This is probably true though traces of gas were obtained after 30 minutes reaction due perhaps to a slight conversion into creatinine. Creatinine, however, reacts rapidly and produces a quantity of gas equivalent to one nitrogen in about an hour, and slightly more in longer experiments. In the first 3 minutes from 15 to 20 per cent of one nitrogen is liberated. Both creatinine and creatinine zinc chloride were studied.

It was thought that the presence of creatinine might account for a considerable part of the relatively large blank which must be subtracted in determinations of amino nitrogen in urine by Van

Slyke's method. In a few experiments it was found that about one-third of the amount of the blank could be accounted for by the reaction of creatinine present.

The influence of temperature on the rate of reaction of three of the compounds was studied. Van Slyke (3), Sure and Hart (4), and Dunn and Schmidt (5) have found that the rates of reaction of nitrous acid with amino groups in amino-acids are functions of the temperature. Creatinine yields in 30 minutes at 21° a quantity of gas equivalent to 89 per cent of one nitrogen, while at

TABLE IV.  
*Effect of Temperature on the Rate of Reaction.*

Quantity of one reacting nitrogen.	Time.	Gas volume corrected.	Temper- ature.	Barometer.	Weight of N found.	Percentage of one reacting nitrogen.
Creatinine.						
mg.	min.	cc.	°C.	mm. Hg	mg.	
0.776 N	30	1.22	21.2	764	0.694	89
	30	0.55	7.8	771	0.335	43
	30	0.47	7.0	771	0.288	37
	30	0.33	2.5	772	0.207	27
	3	0.0	2.0			0
Guanine.						
0.698 N	30	1.31	23.0	763	0.737	106
	30	0.36	2.4	772	0.226	32
Guanosine.						
0.698 N	30	1.54	24.5	757	0.854	122
0.693 N	30	0.80	2.5	772	0.502	72

2.5° it yields only 27 per cent in the same length of time. At about 2° no reaction was detected in 3 minutes. The rates of reaction of guanine and of guanosine are similarly influenced by changes in temperature, though that of guanosine seems to be less affected.

#### SUMMARY.

Determinations of amino groups reacting slowly with nitrous acid were carried out by the use of Van Slyke's method. The

rates of reaction were determined at 21–26° over periods of from 3 minutes to 5 hours. Guanine, guanosine, and guanylic acid yield in 1 to 2 hours amounts of gas varying from 8 to 27 per cent above the theoretical for one reacting nitrogen. Adenine and adenine nucleotide both yield the theoretical amount of nitrogen in 1 to 2 hours. Xanthine and hypoxanthine produce traces of gas, but uric acid does not.

Cytosine yields 37 per cent more than the quantity of gas produced by one reacting nitrogen. Thymine, uracil, and uridine do not react with nitrous acid. 2 atoms of nitrogen in allantoin react slowly and continuously, the reaction not being complete in 5 hours.

1 atom of nitrogen in creatinine reacts with nitrous acid in 1 hour. The presence of creatinine may account for one-third of the blank in the determination of amino nitrogen in urine by Van Slyke's method.

The rates of the reactions are greatly influenced by the temperature.

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## THE DETERMINATION OF FREE AMINO NITROGEN IN PROTEINS.

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In an effort to determine the free amino nitrogen in Bence-Jones protein by the method of Van Slyke (1), it was observed that the protein was precipitated as soon as it entered the reacting chamber. As Bence-Jones protein is relatively insoluble in weakly acid solution, the possibility of an incomplete reaction between the free amino groups and the nitrous acid made it advisable to check the analysis by the formol titration method of Sörensen (2). This led to a comparison of the two methods.

Both methods have been used extensively in the past for the determination of free amino groups. While each has seemed to be fairly satisfactory, difficulties arise in their application under certain conditions. When the individual amino-acids are studied, it is found that, with few exceptions, the same grouping is determined by both methods; *i.e.*, the  $\alpha$ -amino nitrogen. The  $\epsilon$ -amino nitrogen of lysine is also determined although it reacts somewhat more slowly in Van Slyke's procedure than do  $\alpha$ -amino groups. The nitrogen of proline is determined by Sörensen's method, but not by Van Slyke's. Some anomalous reactions are obtained. Glycocoll and cystine give more than the theoretical quantity of gas by Van Slyke's method. Sörensen obtained values higher than the theoretical with tyrosine and lower with histidine, proline, and lysine.

In Sörensen's method the reaction of the protein solution is first adjusted so that the free amino groups of the protein are just neutralized by acid groups, then formaldehyde is added and, by the formation of methylene-amino derivatives, the basic property of the amino groups is destroyed and there is liberated

thereby an equivalent amount of acid which is subsequently titrated. The method has the inherent disadvantage of depending upon the titration, between rather arbitrarily chosen end-points, of mixtures of weakly acidic and basic groups of unknown strength and variable concentration. Theoretically the titration should be made from the isoelectric point of the protein to the end of the titration curve of the compound formed by formaldehyde. But even without the exact conditions of titration defined, the method nevertheless yields results which are probably not far from the theoretical.

In Van Slyke's method the uncertainties of the above titration are overcome by actually measuring the gas liberated by the interaction of the amino groups with nitrous acid, but other difficulties are encountered. Most proteins or their deaminized products precipitate in the nitrous acid solution. It would seem, therefore, that occlusion of some of the material not yet acted upon must occur and cause an increase in the length of time necessary for complete reaction. Van Slyke and Birchard (3) recognized this difficulty and attempted to overcome it by increasing the time of reaction from 2 to 5 minutes to 20 to 30 minutes. Owing to the lack of theoretical values to show when the reaction is complete and owing to the possibility of some hydrolysis of the protein occurring during the time of reaction, it is uncertain whether or not this procedure yields correct results.

Several investigators, working mainly with digestion products of proteins, have compared the two methods. White and Thomas (4) found that the results with Van Slyke's method (apparently using the 5 minute period of reaction) were parallel with, but lower than, those with Sörensen's method. Rogoziński (5) and Andersen (6) noticed variations with the Van Slyke method and concluded that Sörensen's method was more satisfactory. Kossel and Cameron (7) obtained different quantities of nitrogen with Van Slyke's method when sturine was allowed to react for different lengths of time, but later Kossel and Gawrilow (8) made use of the formol titration.

Abderhalden and Kramm (9), in analyzing digestion mixtures of proteins by Van Slyke's method, found that great differences in results were obtained depending on whether the reaction was allowed to continue 5 minutes or 10 and suggested that some

easily split peptones might be hydrolyzed. They were cognizant of the fact that no splitting had been noticed in previous work on pure polypeptides, but considered that these data were insufficient. By running comparative experiments with the formol titration method they found that Van Slyke's method yielded lower results after a reaction of 5 minutes and higher results after a reaction of 10 minutes and suggested that Sörensen's method should be used to standardize the time of reaction of the Van Slyke procedure.

Northrop (10) states: "For absolute determinations of the amino-acids Van Slyke's method is more accurate, for comparative experiments concerning the changes occurring in gelatin solutions, such as were used in this work, the formol titration is more accurate and also much more rapid."

The possibility of hydrolysis of the protein during the determination was considered by Van Slyke and Birchard (3) who concluded that none occurs because:

"1. Peptides of varied composition and containing up to fourteen amino-acids in the molecule have been analyzed by our method and found to give theoretical results.

"2. The evolution of nitrogen is complete inside of twenty or thirty minutes, following practically the course found in analysis of lysine...."

Data concerning the latter statement are considered below.

Hydrolysis can only be studied satisfactorily by eliminating a confusing element in the determination of native proteins; namely, precipitation by nitrous acid in the reacting chamber. Peptone and proteose solutions are useful in this connection as they yield little or no precipitate under the conditions of the experiment. Rice (11) reported a few experiments with peptone to show that not only is more nitrogen obtained in 8 than in 6 minutes, but that the temperature at which the reaction is carried out influences the results, more nitrogen being obtained at the higher temperature. The difference observed between the highest and lowest results is too great to be accounted for by the delayed reaction of the  $\epsilon$ -amino group of lysine, and suggests that there may have been a slight progressive hydrolysis.

Hydrolysis may not be the only factor involved in the increase of nitrogen with the lengthening of time of reaction. Slowly

reacting groups may possibly contribute to the high results. Kossel and Cameron reported that arginine and nitroarginine give off increasing quantities of nitrogen as the time of reaction is increased, quantities which are above the theoretical for one reacting nitrogen. They also found that nitroguanidine gives off small but increasing quantities of nitrogen in the reactions. The author has consistently obtained quantities of gas equivalent to 1 per cent of the total nitrogen by allowing guanidine and methyl guanidine to react for  $\frac{1}{2}$  to 1 hour.

It would appear from this literature that Van Slyke's procedure may possibly yield high results due to hydrolysis and other factors, or low results due to incomplete reaction of the precipitated protein. Sörensen's method on the other hand is not free from theoretical objections. The method of Harding and MacLean (12) seems to be liable to greater errors.<sup>1</sup> Parallel determinations were therefore made on various solutions of proteins using the methods of Van Slyke and of Sörensen for comparison.

The micro apparatus of Van Slyke was used in these analyses. The periods of time allowed for the reaction were based on the suggestion of Van Slyke (14); namely, 3 minutes for temperatures between 20 and 25°;  $2\frac{1}{2}$  minutes between 25 and 30°; and 2 minutes above 30°. Besides these minimum periods of reaction, samples of each solution were allowed to react twice and five times as long. When determinations involving the longest period were carried out, the reacting chamber was shaken vigorously at intervals of about 1 minute to keep the contents well mixed. The blanks for the various reagents varied in different experiments either due to the use of different quantities or different preparations of octyl alcohol. The blanks are not specifically reported, but may be found by subtracting the corrected from the observed readings given in the tables.

The method of Sörensen was carried out as follows: A portion of the protein solution was titrated to pH 7.0 with neutral red as an indicator, using a standard solution for comparison. To another portion of 20 cc. were added 10 cc. of neutralized formalin (40 per cent) and 6 drops of 1 per cent phenolphthalein and the solution was titrated with 0.1 N sodium hydroxide to a deep red

<sup>1</sup> Folin's method (13) was not available when these experiments were carried out.

color. The end-point was matched against the color produced by mixing 20 cc. of neutral boiled water, 10 cc. of neutralized formalin, 0.3 cc. of 0.1 N sodium hydroxide, and 6 drops of 1 per cent phenolphthalein. Where the original protein solution was colored, the standard was placed behind some of the protein solution, similarly diluted, in a comparator and the resulting color was used as the end-point for the titration. From these titrations the quantity of alkali necessary to titrate the solution from pH 7.0 to the end-point chosen was determined, and, after subtracting the 0.3 cc. blank, the amino nitrogen per cubic centimeter was calculated. The total nitrogen was determined by the Kjeldahl method. Duplicates were run in all analyses unless otherwise stated.

Various preparations of protein material were studied, but no attempt was made to get preparations of highest purity with which to make the comparisons. The following materials were used:

1. *Commercial Peptone.*

2. *Proteose 1.*—This was prepared from beef which had been digested with pepsin, by precipitating with ammonium sulfate and removing the salt by boiling with barium carbonate. The filtered solution was concentrated, precipitated with alcohol, and dried with alcohol and ether.

*Proteose 2.*—A portion of Preparation 1 was reprecipitated twice with ammonium sulfate and evaporated *in vacuo* with barium carbonate several times. When free from ammonia it was precipitated with alcohol and dried with alcohol and ether. These preparations gave no test for ammonia by shaking with permutit, and treating it with sodium hydroxide and Nessler's solution.

3. *Egg Albumin, Crystalline.*—The egg albumin crystallized three times, dialyzed, and dried. A trace of ammonia was removed by shaking with permutit.

4. *Egg Albumin, Purified.*—Egg white was diluted with 5 volumes of water and filtered. The albumin was twice precipitated by saturating the solution with sodium sulfate. The final precipitate was dissolved in water and analyzed.

5. *Serum Globulin.*—This was roughly prepared from pig serum by precipitation with sodium sulfate. The globulin precipitate was washed and reprecipitated. The moist precipitate was dissolved and analyzed.

6. *Edestin.*—Edestin was prepared from hemp seed and crystallized three times. It was dried with alcohol and ether, and dissolved in 10 per cent sodium chloride.

7. *Bence-Jones Protein (No. R 5).*—This was twice precipitated with sodium sulfate and acetic acid and dried with alcohol and ether. This

preparation (No. R 5) was isolated from urine which had been preserved with toluene and allowed to stand for 2 or 3 months at room temperature.

In Table I will be found data obtained by analyzing the various solutions of protein material with the methods of Sörensen and of Van Slyke. With the latter method the reactions were permitted to continue for various lengths of time. It is at once apparent that there is considerable disagreement between the two groups of data. There is a continuous increase in the values obtained by Van Slyke's method as the period of reaction is lengthened and no single period of reaction yields results with all of the preparations similar to those obtained by Sörensen's method.

TABLE I.

	Amino nitrogen.				
	Sörensen method.	Van Slyke method.			
		2 to 3 min.	4 to 6 min.	10 to 15 min.	30 min.
	mg. per cc.	mg. per cc.	mg. per cc.	mg. per cc.	mg. per cc.
Peptone.....	0.730	0.648	0.712	0.762	0.790
" *	0.180	0.167	0.188		
Proteose 1.....	0.170	0.158	0.182	0.191	0.200
Egg albumin (crystalline).....	0.127	0.063	0.091	0.104	
" "	0.155	0.077	0.107	0.145	
Serum globulin.....	0.189	0.137	0.170	0.205	
Edestin.....	0.052	0.042	0.045	0.059	
Bence-Jones Protein 5.....	0.138	0.069	0.097		

\* The first solution diluted with 3 volumes of water.

Periods of reaction of 8 to 10 minutes and 20 to 30 minutes have been suggested for analyzing protein material by Van Slyke's method and, as mentioned above, Abderhalden and Kramm used the method of Sörensen to standardize the period of reaction. In the present experiments with the native proteins, edestin and serum globulin, the 4 to 6 minute period of Van Slyke's method yielded lower results than Sörensen's method. With the egg albumin preparations, however, even the 10 to 15 minute periods of Van Slyke's method yielded results lower than the Sörensen procedure. The Bence-Jones protein reacted at a similar rate. On the other hand, the proteose and the dilute

peptone solutions gave higher results in 4 to 6 minutes. These data do not support the idea that any single period of reaction in Van Slyke's method yields results similar to Sörensen's for all types of protein material.

As Van Slyke has observed, the native proteins were precipitated as soon as their solutions were mixed with the acid nitrite solution. This precipitation probably decreased the rate of reaction. In order to determine whether or not the continuous rise in values as the time of reaction increased was due merely to the delayed reaction of material occluded in the precipitate, the solutions of peptone and proteose were carefully studied. The peptone did not precipitate in the reacting chamber and the proteose formed first a turbidity and then, as the time of reaction continued, a slight precipitate. It will be seen that in spite of the lack of a precipitate to retard the action, the values for amino nitrogen increased continuously as the time of reaction was lengthened.

It should be noted, however, in studying the rising values that the  $\epsilon$ -amino group of lysine reacts more slowly than the  $\alpha$ -amino groups of the amino-acids. Some of the free amino nitrogen in the proteose and peptone solutions is undoubtedly  $\epsilon$ -amino nitrogen of lysine. Van Slyke first demonstrated quantitatively that  $\epsilon$ -amino groups of lysine alone are uncombined in native proteins, while in proteoses and peptones, other amino groups are free. As the data reported in this paper show that the peptone preparation contained about 27 per cent of the total nitrogen in the form of free amino nitrogen, the proteose about 8 per cent, and the native proteins 2 to 5 per cent, one may assume that about half of the free amino nitrogen of the proteose and about one-sixth of the free amino nitrogen of the peptone is  $\epsilon$ -amino nitrogen of lysine.

Considerable data concerning the rate of reaction of the  $\epsilon$ -amino group of lysine are now available. Van Slyke reported that at  $24^\circ$ , 95 per cent of the nitrogen reacts in 5 minutes and 100 per cent reacts in 15 minutes; at  $20^\circ$  it reacts completely in 30 minutes. Sure and Hart (15) showed that the reaction is greatly influenced by temperature and that it is complete in 5 minutes at  $32^\circ$ . Dunn and Schmidt (16) have recently reported that the epsilon group of lysine reacts completely in 8 minutes at temperatures between  $26$  and  $30^\circ$ .

Solutions of peptone were studied by Van Slyke's method at temperatures above 30° and in periods of 2, 4, and 20 minutes. A single determination was made using a reaction time of 30 minutes. At the beginning of Table II may be found the individual analyses of these solutions. The reaction should have been nearly complete in 2 minutes at 31° as most of the reacting nitrogen was  $\alpha$ -amino nitrogen. It will be observed that the values rise steadily. A small quantity of ammonia was present in this preparation, but it is doubtful if the continuous increase can be ascribed to it. Values for the 10 and 30 minute periods are higher than those of Sörensen's method with which ammonia is determined completely. A much more probable explanation is that a slight hydrolysis occurs in the Van Slyke procedure yielding results which are too high.<sup>2</sup> This peptone solution was diluted with 3 volumes of water and the diluted solution reacted more rapidly than the stronger solution, yielding results in 4 minutes practically as great proportionately as yielded by the stronger solution in 10 minutes. Sörensen's method yielded the expected values with the diluted solution, a value slightly less than that given by Van Slyke's method in 4 minutes.

Proteose solutions yielded results similar to the peptone solutions. The values increased as the time of reaction was lengthened. Solution 1 in 6 minutes gave higher results by Van Slyke's method than by Sörensen's. Solution 2, which was more dilute and was analyzed at a higher temperature yielded values which increased even more rapidly. If the 30 minute period is assumed to yield the correct result, then in 2 minutes only 60 per cent of the amino nitrogen had reacted instead of 90 to 95 per cent which would be expected from reactions of amino-acids and polypeptides. Such a calculation is obviously incorrect and leads to the conclusion that the long periods of reaction yield results which are too high. A slow continuous hydrolysis appears to be the cause of the high values.

<sup>2</sup> Another explanation, of course, is that there are unknown groups in these preparations which react slowly with nitrous acid at a rate even slower than that of the  $\epsilon$ -amino group of lysine. These slowly reacting groups are presumably not determined by Sörensen's method because the results obtained with this method are lower than the maximum values observed with Van Slyke's method.

TABLE II.

Time.	Temperature.	Reading.	Corrected reading.	NH <sub>2</sub> -N per cc.	Percentage of highest value.
Peptone.					
2 cc. of 2 per cent solution used with 0.10 cc. octyl alcohol. Barometer 755 mm. Hg.					
min.	°C.	cc.	cc.	mg.	per cent
2	30	2.54	2.42	0.648	82
4	31	2.80	2.67	0.712	90
10	31	3.00	2.86	0.762	96
30	30	3.13*	2.95	0.790	100
2 cc. of 0.5 per cent solution used with 0.10 cc. octyl alcohol. Barometer 754 mm. Hg.					
2	31.5	0.75	0.63	0.167	89
4	31.9	0.84	0.71	0.188	100
Proteose.					
1.5 per cent Proteose 1, 2 cc. used with 0.10 cc. octyl alcohol. Barometer 760 to 762 mm. Hg.					
3	25.5	0.69	0.57	0.158	79
6	26.5	0.79	0.66	0.182	91
15	26.0	0.83	0.69	0.191	95
30	27.5	0.91*	0.73	0.200	100
Proteose 2, 2 cc. used with 0.10 cc. octyl alcohol (different preparation). Barometer 776 mm. Hg.					
2	32.0	0.46	0.33	0.090	60
5	31.0	0.57	0.43	0.118	79
15	32.0	0.65	0.49	0.134	89
30	32.0	0.75	0.55	0.150	100
Serum globulin.					
2 cc. used with 0.15 cc. octyl alcohol. Barometer 762 mm. Hg.					
2	32	0.66	0.51	0.137	67
4	33	0.80	0.64	0.170	83
10	33	0.94	0.77	0.205	100
Crystalline egg albumin.					
2 cc. used with 0.15 cc. octyl alcohol. Barometer 760 mm. Hg.					
2	27.6	0.38	0.23	0.063	61
4	27.0	0.49	0.33	0.091	87
10	27.8	0.55	0.38	0.104	100

\* Single determination.

TABLE II—Concluded

Time.	Temperature.	Reading.	Corrected reading.	NH <sub>2</sub> -N per cc.	Percentage of highest value.
Purified egg albumin.					
2 cc. used with 0.15 cc. octyl alcohol. Barometer 758 mm. Hg.					
min.	°C.	cc.	cc.	mg.	per cent
2½	26.5	0.43	0.28	0.077	53
5	26.8	0.55	0.39	0.107	74
13	27.0	0.70	0.53	0.145	100
Edestin.					
2 cc. used with 0.10 cc. octyl alcohol. Barometer 764 mm. Hg.					
3	24	0.27	0.15	0.042	71
6	24	0.29	0.16	0.045	76
15	24	0.35	0.21	0.059	100
Bence-Jones Protein 5.					
2 cc. used with 0.10 cc. octyl alcohol. Barometer 762 mm. Hg.					
3	25	0.37	0.25	0.069	71
6	25	0.48	0.35	0.097	100

The method of Van Slyke seems, therefore, to be subject to error in two directions when used in analyzing protein material: (1) by yielding results which are too high, due probably to hydrolysis; and (2) by yielding results which are too low with certain proteins which are too insoluble in the nitrous acid. Less objection can be raised to the use of Sörensen's method with the proteins studied. No precipitate is formed to render the solution difficult to handle, and the titration can be carried out without difficulty even with colored solutions.

#### SUMMARY AND CONCLUSIONS.

The methods of Sörensen and of Van Slyke for the determinations of free amino nitrogen have been compared, using solutions of native and derived proteins.

The method of Van Slyke yielded results which varied depending on the length of time of reaction and the material examined. Hydrolysis may account for high results and precipitation of the protein material by the reagent may account for some low results.

The method of Sörensen seems to be less susceptible to error in the analysis of proteins.

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## A SPONTANEOUS CRYSTALLIZATION OF A BENCE-JONES PROTEIN.

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Many observations have been made on the protein material designated as Bence-Jones protein which is found occasionally in urine from patients suffering from certain disorders. Attempts to crystallize some of these preparations have rarely been successful and then usually only by accident. Below is reported an observation of spontaneous crystallization which is unique in two respects. It is apparently the first time that a Bence-Jones protein has been observed to crystallize spontaneously from the urine as passed and, with one exception, is the only observation of any protein material crystallizing spontaneously from urine.

Henry Bence-Jones reported in 1847 (1) the peculiar reactions of the protein material which now is designated by his name. He stated that 2 years previously there was found in urine from a patient with osteomalacia a protein which gave no precipitate with nitric acid in the cold except on long standing, but when the solution was heated and allowed to cool it became solid. The precipitate redissolved on heating and formed again on cooling.

Heller (2) described somewhat similar observations in a text-book bearing the date of 1846. He had found several cases where the urine contained protein material which he recognized to be different from albumin and casein. It coagulated at about 50°, but the addition of small amounts of nitric acid completely hindered the coagulation by heat. The most unique property of Bence-Jones protein, solubility at the boiling point and reprecipitation on cooling, was not mentioned.

Similar observations were not made for many years. Kühne (3) in reporting a case which was observed in 1869 states that Bence-Jones had written in a personal communication that he had not seen another case of Bence-Jones proteinuria between 1845 and 1869. Within recent years, however, a number of cases have been reported.

Spontaneous precipitation of an amorphous precipitate of Bence-Jones protein seems to be not uncommon. Bradshaw (4) states that a patient under his observation passed, two or three times a week, milky urine which deposited a copious amorphous sediment giving protein reactions. Rosenbloom (5) reported a case where an amorphous precipitate of Bence-Jones protein occupied one-third of the volume of each specimen of urine. Several authors report milky urine while others record that the protein did not precipitate on standing. Several of the urines which I have examined deposited an amorphous precipitate of protein after standing several weeks.

Magnus-Levy (6) attempted to crystallize a Bence-Jones protein and accidentally obtained crystals from one preparation after standing for months. He was unable to repeat the experiment. Grutterink and de Graaff (7) studied a case of Bence-Jones proteinuria and succeeded in causing the protein to crystallize. They precipitated the protein with 2 volumes of saturated ammonium sulfate and dialyzed a solution of the precipitate for 4 days. To the resulting solution was added one-ninth its volume of saturated ammonium sulfate, an equal volume of water, and a few drops of sulfuric acid. Crystals formed within 24 hours. They were said to be too insoluble to recrystallize.

Schumm and Kimmerle (8) report that they accidentally obtained crystals of a Bence-Jones protein on one occasion under very peculiar conditions. Some sodium chloride and acetic acid were added to urine containing a Bence-Jones protein, the mixture was boiled and filtered hot. The protein in the filtrate was precipitated with alcohol, washed once, and the damp precipitate dissolved with the aid of heat in water containing a little acetic acid. After filtering, the turbid solution was placed in an ice box. The next morning, a small quantity of crystals was found which contained but little ash and responded to all of the simple tests for Bence-Jones protein.

Since the preliminary report (9) was made of the preparation described below, Krauss (10) has succeeded in crystallizing a Bence-Jones protein. Many liters of urine were half saturated with ammonium sulfate. The precipitate was dissolved in water, the solution dialyzed until free from ammonia and then allowed to evaporate at room temperature. After several weeks crystals appeared.

Loehlein (11) reported finding numerous crystals, having the forms of needles, prisms, and plates, in the tubules of a kidney at autopsy from a case of myeloma which had excreted Bence-Jones protein. The crystals were not soluble in liquid solvents; they swelled in dilute alkali, were anisotropic, and stained with dyes which indicated that they were protein. This is the first report of what may have been spontaneously crystallized Bence-Jones protein.

Glaus (12) observed myeloma cells in which there were crystal-like needles and Hedinger (13) states that he had observed a similar phenomenon.

A protein which crystallized spontaneously from the urine was reported by Bramwell and Paton (14). It crystallized from the urine often within 2

or 3 days and some times not for several weeks. They concluded that it was a globulin. Huppert (15) thought from the description that it might be a Bence-Jones protein, but after studying some of the material (16) agreed that it was a true globulin and quite dissimilar from the Bence-Jones protein.

We may state then that up until the present time a spontaneously crystallized Bence-Jones protein has not been observed with certainty. Several cases are recorded where crystallization was accomplished by artificial means. The spontaneously crystallized Bence-Jones protein described below seems, therefore, to be unique.

A patient at the Mayo Clinic<sup>1</sup> thought to-be excreting Bence-Jones protein was transferred to Dr. Rountree's service for special study. My attention was called by Dr. Rountree to the peculiar milkiness which developed in the urine from this patient soon after it was passed. Under the high power of the microscope there was a slight suggestion that the precipitate might be crystalline. The precipitate showed on the next day a more definite crystalline structure, in appearance similar to freshly crystallized egg albumin. The turbid fluid also showed a marked sheen upon being shaken.

Some of the crystalline material was separated by centrifugation, washed once with cold water, and dissolved in water by adding a trace of sodium hydroxide. The solution was acidified slightly with acetic acid, a small quantity of sodium chloride added, and the whole heated slowly. The protein began to coagulate at 57°. Coagulation seemed to be complete at about 70°. The precipitate dissolved almost completely between 80-85°, leaving a slightly opalescent solution which did not clear up on boiling. The precipitate reappeared as the solution cooled to 80°. Heating and cooling again caused the same phenomena.

The acid urine gave the same reactions. In the more concentrated solutions of the crystalline material, as well as the urine itself, the precipitate dissolved on heating, leaving a small residue which was gummy and stuck to the sides of the test-tube. Magnus-Levy (6) reported the same phenomenon. From these

<sup>1</sup> The case was referred by Dr. Eusterman to the service of Dr. Rountree and was reported by Dr. Walters (17). My investigations were begun in Rochester and completed in Baltimore.

preliminary observations it seemed justifiable to conclude that the patient was excreting a Bence-Jones protein which crystallized spontaneously on standing a few hours.

In order to obtain a quantity of material, the urine was collected, preserved with toluene, and allowed to stand at room temperature. The volume of urine in 12 hours varied from 300 to 1,000 cc. It was usually acid and was always clear when voided and contained no casts. About 15 gm. of protein were excreted per day. Some time later metabolic experiments were carried out by Walters (17) who found the excretion varying from 10 to 28 gm. daily. Spontaneous crystallization occurred after varying intervals, depending apparently on the concentration and acidity of the urine. In one instance crystallization occurred within 8 hours after the 12 hour specimen had been completed.

On standing the crystals increased in size until they could be easily seen by the low power of the microscope. The largest crystals appeared as long rectangular plates which were very thin. They were so thin that, while they seemed to be doubly refractive, the observation was somewhat doubtful.

After the urine had stood several days and crystallization seemed to be complete, a considerable quantity of protein still remained in solution. The dissolved protein coagulated at the same temperature as a solution of crystals. Attempts were made to bring about a more complete crystallization from the urine artificially. By experimenting on portions of a concentrated urine it was found that no crystals formed in neutral or alkaline solutions. Crystallization occurred readily when the urine was made acid to litmus with a small quantity of acetic acid, while no crystals formed when the urine was made as acid as pH 4. No crystals were obtained when ammonium sulfate, ammonium chloride, or sodium chloride were added in amounts to form varying quantities of precipitate, even when the tubes were seeded with crystals previously obtained. These reagents seemed to prevent crystallization completely. Long standing in the ice box and at room temperature failed. But the crystalline material could easily be recrystallized by dissolving in water made slightly alkaline with sodium hydroxide, acidifying with acetic or sulfuric acid, and allowing to stand over night. Crystals formed more rapidly when the solutions were seeded.

In order to obtain a dry preparation of this protein, Taylor and Miller's method (18), slightly modified, was used.

To 100 cc. of filtered urine were added 15 cc. of supersaturated sodium sulfate solution (200 gm. of crystalline sodium sulfate dissolved in 100 cc. of warm water) and 20 cc. of glacial acetic acid. After standing 15 to 20 minutes, the solution was centrifuged and the clear supernatant fluid decanted. The precipitate was washed by centrifugation once with acidulated water, once with 50 per cent alcohol, twice with 95 per cent

alcohol, once with absolute alcohol, once with absolute alcohol and ether, and finally with ether. It was then suspended in ether, transferred to a Buchner funnel, and pumped dry. A fine, colorless powder was obtained. A fresh 24 hour specimen of urine, 1,370 cc. in volume, yielded 15 gm. of air-dry protein.

Attempts were made to crystallize some of this dry preparation. The procedure which was successful in recrystallizing the moist crystals was entirely unsuccessful when applied to the dried preparation.

A quantity of crystals from urine was centrifuged, then washed with alcohol and ether as described above. This dry preparation yielded small quantities of crystals when recrystallization was attempted. The yields were so small, however, that it was concluded that the drying with alcohol and ether damaged the preparation.

After some experimentation the following procedure was adopted:

The supernatant liquid was decanted from the crystals which formed in the urine on standing. The remaining fluid was centrifuged, the liquid decanted, and the colorless crystals were suspended in four times their volume of acidulated water and centrifuged again. After decanting the supernatant liquid, the crystals were dissolved in a small amount of water with the aid of a few drops of sodium hydroxide, and the solution was filtered. The filtration was slow. The slightly opalescent filtrate was acidified with acetic acid and a drop of crystal suspension was added. The fluid became turbid in about 2 minutes and within 5 minutes it was milky white. Under the microscope small wart-like masses of needle plates could be seen. After standing over night, the suspension was centrifuged. The supernatant liquid contained considerable protein, but no further crystallization occurred on standing in the ice box. The crystals were again dissolved and recrystallized from a nearly colorless solution. On standing over night, large burrs were observed which broke up easily. See Fig. 1. After centrifuging, the cream of crystals was transferred to a porous plate, spread into a thin layer, and allowed to dry partially. When nearly dry, the now transparent film (crystals had disappeared) was scraped off. It was allowed to dry and then ground to a powder. 9 gm. of material were prepared in this way. This preparation could be crystallized easily by dissolving in water with a trace of alkali, then acidifying, and allowing to stand over night.

The recrystallized preparation gave all the typical reactions of Bence-Jones protein. A comparison was made between the

coagulation temperatures of the original urine, the sodium sulfate preparation, and the recrystallized preparation. They all reacted similarly. Coagulation began between 47 and 52°, depending on the concentration of protein and concentration of salts and acid. The coagulum dissolved at about 80°, leaving a slight residue which did not dissolve even when a dilute solution was boiled. If the solution were cooled quickly, a considerable precipitate formed.

Some studies were made to analyze the conditions influencing the crystallization of the precipitated Bence-Jones protein. The preparations isolated by the use of sodium sulfate and glacial

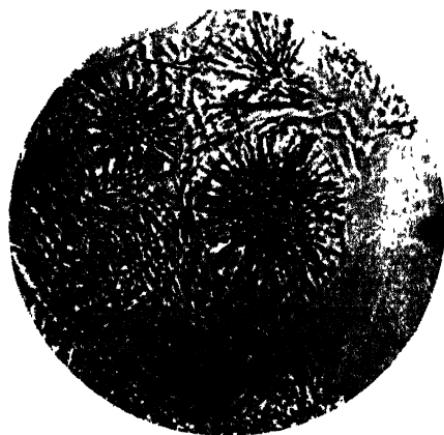


FIG. 1. Bence-Jones protein. Recrystallized three times.

acetic acid, as described above, could not be crystallized. When smaller quantities of acetic acid were used in the precipitation, some crystallization occurred. 25 cc. of urine were precipitated with 12.5 cc. of sodium sulfate after adding 3 cc. of glacial acetic acid. The pH was roughly 5. The precipitate was removed by centrifugation and dissolved in a small quantity of water with the aid of a little sodium hydroxide. The solution was slightly acidified, seeded, and allowed to stand at room temperature. A small quantity of crystals was obtained after several days.

Experiments were made acidifying the urine with sulfuric acid instead of acetic and precipitating with sodium sulfate. Good

yields of crystals were obtained quickly when from 5 to 10 drops of 10 per cent sulfuric acid were added to 25 cc. of urine, the most acid solution being neutral to methyl orange. When 15 drops were added ( $\text{pH} \pm 3$ ) the yield was much poorer and the crystallization slower. Too much acid seemed to damage the protein and decrease the ease of crystallization. Ammonium chloride and sulfate could be used just as well as sodium sulfate. When ammonium sulfate or sodium sulfate with sulfuric acid was used, the precipitates were highly colored. When ammonium chloride or sodium sulfate with acetic acid was used, the precipitates were nearly colorless.

Dialysis of the solutions of the precipitates seemed to aid crystallization. But dialysis of the mother liquor from which crystals had been obtained caused no further crystallization even when the solution contained a large quantity of protein which gave the typical reactions for Bence-Jones protein.

After these experiments were carried out, many attempts were made to crystallize the Bence-Jones proteins from various sources. The method of Grutterink and de Graaff was also used. The experiments were uniformly unsuccessful.

The blood of the patient who excreted the crystallizable material was examined for Bence-Jones protein.

15 cc. of serum were diluted with 75 cc. of water and 1 gm. of sodium chloride was added. 40 cc. of this solution were taken, heated to the boiling point after adding a few drops of acetic acid, and filtered through a heated funnel into a hot flask, the filtrate being kept near the boiling point. The filtrate was slightly acid and water-clear. It was allowed to cool and at about  $75^\circ$  a marked turbidity developed which increased in density on further cooling; but did not form a flocculent precipitate. The solution was again heated and the precipitate dissolved almost entirely, leaving the solution only faintly opalescent. A precipitate started to form at about  $85^\circ$ . The solution was placed in a water bath at  $60^\circ$  and kept between 55 and  $65^\circ$  for 4 hours. It was then centrifuged and the supernatant liquid which was slightly opalescent was decanted. The precipitate was washed once with 50 per cent alcohol and dried at  $100^\circ$ . 3.8 mg. of dry protein were obtained which was equivalent to 0.057 gm. per 100 cc. of blood. A second determination yielded 0.039 gm. per 100 cc. These values are certainly minimum values because the total quantity of Bence-Jones protein does not precipitate after boiling.

Normal serum was treated in the same way and no trace of opalescence appeared on cooling the hot filtrate.

Many isolated studies of the properties of Bence-Jones protein have been carried out, but preparations from different sources have not often been compared, under identical conditions. Different investigators have reported coagulation temperatures varying from 40 to 60°. The solutions some times became clear on boiling and some times not. Hopkins and Savory (19) pointed out the enormous effect of the presence of neutral salts on the precipitation and solution with heat. The temperature at which the protein coagulates varies also with the concentration of protein and the acidity.

In order to minimize the effects of these variables, the urines from several cases were diluted with normal urine until only a slight cloud formed on heating. Portions of 5 cc. were acidified with 5 drops of 10 per cent acetic acid and heated slowly in the same water bath. The temperatures at which precipitation and solution occurred were noted.

	R (X5)	CND (X10)	JED (X4)	RL (X50)
	°C.	°C.	°C.	°C.
Precipitate forming.....	51	51	76	59
"    maximum.....	56	56	82	60
"    dissolving.....	89	89	96	99
"    dissolved.....	98	98	98	99

The figures in parenthesis show the volumes of normal urine used in diluting the pathological urines.

It is at once apparent that considerable variations in the temperature of coagulation and solution may occur among different Bence-Jones proteins. It is interesting to note that while the proteins from R and from CND reacted identically with heat, the two proteins have been shown to be different in other respects. The protein from R crystallized spontaneously and the other could not be crystallized. Immunological studies by Bayne-Jones and the author (20) have also brought out differences between them.

On account of the peculiar physical properties of Bence-Jones protein, many investigators in the past have considered the material to be an albumose. Hopkins and Savory (19) first showed that the material is more nearly similar to the native proteins than to the albumoses because they were able to isolate practically all of the amino-acids usually found in native proteins.

In an attempt to gain added information regarding the nature of the molecule, the free amino nitrogen of the purified preparations of Bence-Jones protein was compared with the total nitrogen. Van Slyke (21) and Van Slyke and Birchard (22) have found that the free amino nitrogen of native proteins varies between 0 and 6 per cent of the total nitrogen, while derived proteins such as albumoses yield from 8 to 10 per cent. An unexpected difficulty which is described in the previous paper<sup>2</sup> was encountered in the use of Van Slyke's method for the determination of free amino nitrogen in Bence-Jones protein. Sörensen's method of formol titration (23) was therefore employed in obtaining the data reported in this paper. Various protein preparations were examined in order to make possible a comparison of results obtained under

TABLE I.

Material.	Free amino N.	Total N.	<u>Free NH<sub>2</sub>-N</u> <u>Total N</u>	
			mg. per cc.	per cent
Peptone, 2 per cent.....	0.730	2.73	26.8	
"    0.5 per cent.....	0.180	0.68	26.4	
Proteose from beef.....	0.170	2.12	8.02	
Bence-Jones protein, No. R 4, crystalline.....	0.139	2.86	4.86	
"    "    No. R 5.....	0.138	2.50	5.52	
Egg albumin.....	0.127	2.49	5.10	
Serum globulin.....	0.189	4.50	4.20	
Edestin.....	0.052	2.70	1.93	

similar conditions. Besides the solutions described in the previous paper, the Bence-Jones protein (No. R 4) was examined. It had been crystallized three times and dried on a porous plate. It was quickly dissolved in water to which had been added 2 or 3 drops of sodium hydroxide and the solution was neutralized as soon as possible.

The solutions were made up, with the exception of the stronger peptone and the edestin, so that similar concentrations of free amino nitrogen would be present in solution. The results obtained with the formol titration are recorded in Table I together with the total nitrogen and the calculated ratio of free amino nitrogen to total nitrogen.

<sup>2</sup> Wilson, D. W., *J. Biol. Chem.*, 1923, lvi, 191.

The crystallized Bence-Jones protein contained 4.86 per cent of the total nitrogen in the form of free amino nitrogen which is slightly less than that in crystallized egg albumin. The proteose preparation contained about 8 per cent free amino nitrogen while the peptone yielded much higher values. The evidence would indicate that the Bence-Jones protein is more nearly similar to the proteins than to the proteoses or albumoses.

Further consideration must be given this evidence before drawing a definite conclusion. The ratio of the free amino nitrogen to total nitrogen was found to be much lower in edestin than in any of the other proteins examined. Similar variations have been reported by several investigators (22, 24, 25). Van Slyke and Birchard (22) were the first to point out that the amount of free amino nitrogen in proteins is very close to half the nitrogen of the lysine present and concluded that the  $\epsilon$ -amino group of lysine is practically the only free amino group in the protein molecule, while other amino groups are free in proteoses.

The lysine content of a Bence-Jones protein had not, until recently, been determined by Van Slyke's method. Hopkins and Savory (19) isolated 3.67 per cent of lysine from a Bence-Jones protein, obtaining 4.32 per cent of the total nitrogen in the form of lysine nitrogen. Grutterink and de Graaff (26), determining the partition of nitrogen by the procedure of Kossel and Kutscher found 8.05 per cent of the total nitrogen in the lysine fraction.

Lüscher (27) in Hopkins' laboratory, using Van Slyke's method for studying the nitrogen distribution, has recently found 8.04 per cent of the total nitrogen in the lysine fraction.

There seems to be enough similarity in the chemical composition between the various specimens of Bence-Jones protein which have been analyzed in this way to permit a rough comparison of them with the crystalline Bence-Jones protein reported in this paper. It was found to contain 4.86 per cent of nitrogen in the form of free amino nitrogen which is not far from one-half of the lysine nitrogen (4.02 per cent) as determined by the analyses reported above. It is not evident why the precipitated preparation (No. R 5) should contain a higher percentage of free amino to total nitrogen unless there was slight hydrolysis on standing for several months in preserved urine before it was isolated.

The free amino nitrogen is somewhat higher than one-half the lysine nitrogen, but the comparison is obviously rough. As the ratio of free amino nitrogen to half the lysine nitrogen is not as great as that found by Van Slyke for albumoses and as the percentage of free amino nitrogen to total nitrogen is similar to a number of other characteristic proteins it seems justifiable to conclude that the Bence-Jones protein is a true protein and not an albumose.

Lüscher states in his recent article: "On the whole, Bence-Jones' protein seems to be a substance, not only characterised by its physical behaviour, but also by its distribution of amino-acids, which differs from all the proteins analysed up to the present time." An examination of his data, however, shows that there is a great similarity between his analyses of Bence-Jones protein and those of the serum globulin studied by Hartley (24). The agreement in the analyses is as good as is met with in comparisons of proteins placed in the same class such as the vegetable globulins studied by Johns and his coworkers (28, 29).

The statement is also made<sup>8</sup> that: "There is some evidence that the same protein appears in the urine in all cases of Bence-Jones' proteinuria." The only evidence presented is that, in two cases, the isolation of amino-acids; and in two cases, a study of the distribution of some forms of nitrogen yielded results which were similar and probably within the errors of the methods used. It is well recognized that these methods can be used only in making relatively rough comparisons of the chemical constitution of similar proteins.

By studying the immunological reactions of Bence-Jones proteins from various sources, Bayne-Jones and the author have obtained data which argue against the conclusion of Lüscher. These methods are far more sensitive than any ordinary chemical methods and seem to depend on the chemical constitution of the protein molecule. The experiments demonstrated that different Bence-Jones proteins do exist and may be obtained from different patients.

#### SUMMARY.

A Bence-Jones protein was found which crystallized spontaneously from the urine in which it was excreted. Dried preparations were made which could be recrystallized.

<sup>8</sup> Lüscher (27), p. 563.

Attempts were made to crystallize other Bence-Jones proteins, but without success.

The crystalline Bence-Jones protein contains a ratio of amino nitrogen to total nitrogen of 4.86 per cent. This is added evidence that the compound should be classed as a protein and not as an albumose.

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## STUDIES IN PYRIMIDINE METABOLISM.

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As purines and pyrimidines are so closely associated in nature it is strange that so little information is available concerning the metabolism of the pyrimidines while a large mass of data has been collected concerning the purines. Steudel (1) carried out some inconclusive experiments on the conversion of pyrimidines into purines. Sweet and Levene (2) observed no change in the output of uric acid of a dog with an Eck fistula after feeding thymine. More than half of the thymine was recovered in the urine. Mendel and Myers (3) studied the fate of the three naturally occurring pyrimidines: thymine, cytosine, and uracil. All three compounds were found to be non-toxic. They appeared to have no influence on the metabolism of urea, purines, or creatinine. The data suggest that they pass through the body and are excreted in the urine unchanged. The authors recovered from the urine of the animals 30 to 50 per cent of the thymine, small quantities of cytosine, and as high as 77 per cent of the uracil that was fed. When one considers the methods of isolation employed it would appear that uracil and perhaps thymine are excreted quantitatively in the urine.

Sweet and Levene were able to recover more than one-half of the thymine which they fed to a dog, but were unable to recover any thymine when it was fed combined in the form of nucleic acid. Mendel and Myers fed 10 gm. of yeast nucleic acid to a rabbit and failed to recover enough uracil or cytosine to give even a sensitive color reaction. A similar experiment was tried with men on a diet high in nucleic acid with similarly negative results.

The presence of pyrimidines in the urine of animals after feeding the free compounds and their absence from urine after feeding pyrimidines combined in the form of nucleic acid suggested a study of the effects of feeding other combinations of the pyrimidines, the hydrolytic products of yeast nucleic acid. Uracil nucleoside and a mixture of cytosine and uracil nucleotides were prepared and fed to rabbits. A single experiment was carried out on man.

The nucleoside was prepared by the method of Levene and Jacobs (4) and the nucleotide preparation was obtained by the procedure of Jones and Read (5). The uracil nucleoside was colorless and pure. The nucleotide preparation was a mixture of cytosine and uracil nucleotides of uncertain purity. It was used because it could easily be prepared in quantity, while the pure nucleotides were much more difficult to obtain. It could be used to advantage because it was free from purine groups and twice as rich in pyrimidine groups as yeast nucleic acid.

The rabbits were kept in metabolism cages, and fed a diet of corn, oats, and carrots in constant daily quantities throughout an experiment. The urine was collected under toluene, in 24 hour periods, the samples being separated as sharply as possible by expressing the urine from the bladders of the animals at a uniform time each morning.

The urines were made up to 300 cc. when the volumes were less than that and portions taken for analysis. The following methods were used: total N, Kjeldahl; urea N, Marshall; ammonia, Folin aeration; creatine and creatinine, Folin; purine N, Krüger and Schmidt; and allantoin, Wickowski. Besides these analyses, some other nitrogen partitions were run to determine whether pyrimidines or pyrimidine derivatives were being excreted.

When uracil was to be isolated, and the nitrogen precipitated, by lead and mercury was to be determined, from 230 to 270 cc. of a day's urine were taken. Lead acetate was first added to the acidified urine and the precipitate filtered off. The filtrate was made faintly alkaline with barium hydroxide and a slight excess of basic lead acetate was added. After filtering, the lead was removed from the filtrate with sulfuric acid and mercuric sulfate added in excess. The mercury precipitate was decomposed with

sulfuretted hydrogen and the resulting solution decolorized with charcoal and evaporated to 1 cc. or less. When uracil was present it crystallized from the solution in characteristic rosettes of needle prisms. It was recrystallized, dried, and weighed. Some specimens of crystals were analyzed for nitrogen. All were tested by the color reaction of Wheeler and Johnson (6) which was carried out as follows: A few milligrams of the solid were placed in a small porcelain dish, a few drops of bromine water were added, and the solution was heated to boiling over a small flame. After cooling, another drop of bromine water and several drops of barium hydroxide solution were added. A deep purple color was produced when uracil was present.

The above method of isolation was always carried out with roughly quantitative precautions so that a total daily elimination could be calculated. The volumes of liquid before and after filtration on a Buchner funnel were noted in each case. Washings of the precipitates were never added to the filtrates. As the precipitates were never very large the calculations based on the portion of original volume taken in each case yield satisfactory figures for comparison. When the nitrogen in a precipitate was to be determined, the precipitate was washed once on the funnel to remove most of the adhering liquid and then decomposed with sulfuretted hydrogen (for the mercury precipitate) or sulfuric acid (for the lead precipitate). The resulting precipitate was carefully washed and an aliquot part of the filtrate taken for analysis.

In Table I are recorded the data of one experiment on a rabbit. After a fore period of 3 days 1.5 gm. of uracil nucleoside were administered subcutaneously. Calculating from the average daily excretion of the fore period there was found a large increase in urea excretion. The undetermined nitrogen was also increased, but the total *extra* undetermined nitrogen which should include pyrimidine nitrogen was little more than half of the nitrogen fed as nucleoside.

Portions of the normal and experimental urines were used for the isolation of uracil. From a portion of the urine obtained on the first day after giving the nucleoside, 67 mg. of uracil were actually isolated (equivalent to 140 mg. of uracil excreted in the total 24 hour specimen), while not even a color reaction was

## Pyrimidine Metabolism

obtained with the residue from the normal urine. The nitrogen in the mercury precipitate of the normal urine was equivalent to 8.5 mg. for the day. On the 1st day after giving the nucleoside 63.6 mg. of N were precipitable by mercury. This accounts for a large part of the extra undetermined nitrogen.

In Tables II, III, and IV, the data from similar experiments are recorded. It should be noted that in every experiment but one a large increase in the elimination of urea was observed. The only evident explanation is that the uracil nucleoside exerts a slight toxic action causing an increase in protein metabolism.

TABLE I.  
*Uracil Nucleoside.*

Rabbit 1. Weight 1,800 gm. 1.5 gm. = 0.170 gm. of N injected subcutaneously on the 4th day.

Day.	Volume. cc.	Total N. mg.	Urea N.		Undetermined N. mg.	Extra total N. mg.	Extra urea N. mg.	Extra undeter- mined N. mg.	N in Hg pre- cipitate. mg.	Uracil. mg.
			mg.	per cent						
1	150	364	232	64	132					
2	165	310	202	65	108					
3	150	327	211	64	116				8.5	0
4	235	507	314	62	193	173	100	75	63.6	140
5	170	526	393	75	133	192	178	15		
6	150	496	367	74	129	162	152	11		
7	195	388	275	71	113	54	60			
8	140	288								

A tendency toward diarrhea and a diminished desire for food also suggest metabolic disturbances.

Table II records a single experiment showing no change in the creatinine excretion. In Table III the variations in the output of creatinine and purines are shown to be unimportant. Traces of creatine were found each day that it was determined. A single allantoin determination on the 1st day after nucleoside ingestion yielded a normal value of 11 mg. of N per day.

In Experiment II, uracil was isolated in both experimental periods and the second preparation was analyzed by the micro

Kjeldahl method. 25.3 per cent of N was obtained; theoretical, 25.03 per cent of N.

The nitrogen in the mercury precipitates from which uracil was isolated accounts for a considerable part of the undetermined nitrogen varying from 54 to over 100 per cent in the

TABLE II.  
*Uracil Nucleoside.*

Rabbit 2. Weight 2,580 gm. 2 gm. = 0.227 gm. of N given *per os* on the 4th day; 1.5 gm. = 0.170 gm. of N given subcutaneously on the 18th day.

Day.	Volume.	Total N.		Urea N.		Creatinine N.	Undetermined N.	Extra total N.	Extra urea N.	Extra undetermined N.	N in basic lead acetate precipitate.	N in Hg precipitate.	Uracil.
		cc.	mg.	mg.	per cent								
1	253	804	579	72	42	225							
2	405	787	586	74	43	201							
3	460	763	573	75	45	190					13.8	2.5	0
4	305	800	560	70	42	240	25		45	11.1	79.4	190	
5	380	674	508	75	45	166							
6	285*	602	469	78	37	133							
14	390	792	583	74		209							
15	365	678	490	72		188							
16	330	698	522	75		176					12.0	2.4	0
17	320	577	426	74		151							
18	270†	1,080	830	77	250	429	351	78	25.3	44.0	95		
19	315	724	548	76	176	73	69	4					
20	370	698	522	75	176	47	43	4					

\* Some urine lost.

† Part of food not eaten.

Uracil analyzed. Obtained 25.3 per cent of N; theory 25.03 per cent of N.

various experiments. But the quantities of nitrogen in the mercury precipitates and the quantities of uracil isolated, make it appear that only a small portion of the uracil fed as the nucleoside was excreted as uracil. Assuming that all the nitrogen in the mercury fraction was uracil nitrogen, the best recovery ac-

## Pyrimidine Metabolism

counts for only 34 per cent of the nitrogen administered. The precipitation of uracil is so nearly quantitative by this method that these results prove that much of the uracil was not eliminated in the urine.

In order to determine whether much of the uracil nucleoside was excreted unchanged, the basic lead acetate precipitate which

TABLE III.  
*Uracil Nucleoside.*

Rabbit 3. Weight 2,800 gm. 2 gm. = 0.227 gm. of N injected subcutaneously on the 4th day; 1.5 gm. = 0.170 gm. of N injected intraperitoneally on the 10th day.

Day.	Volume. cc.	Total N. mg.	Urea N.		Creatinine N. mg.	Purine N. mg.	Undetermined N. mg.	Extra total N. mg.	Extra urea N. mg.	Extra undetermined N. mg.	N in basic lead acetate precipitate. mg.	Uracil. mg.
			mg.	per cent								
1	155	770	602	78			168				9.6	0
2	245	758	577	76			181					
3	220	552	418	76			134					
4	205	1,195	888	74			307	502	356	146	52.0	+
5	160	1,110	896	82			214	417	364	53		
6	140	877	704	80			173	184	172	12		
7	150	792	627	79			165	99	95	4		
8	200	870	687	79	43	18.3	118	177	155			
9	230	573	447	78	30	10.4	85					
10	160*	1,370	1,085	79	56	24.2	200	625	498	77		
11	190	723	563	78	42	11.6	105					
12	215	604	468				136			13		

\* Part of food not eaten.

On the 4th day 11.5 mg. of allantoin N were excreted.

should contain the nucleoside was examined in three experiments (Tables II and III). After decomposing the precipitate as described, the final filtrate was evaporated to a small volume and made up to 10 cc. Portions of this solution were used for various tests. The nitrogen precipitated by basic lead acetate was appreciably higher on the experimental days in one out of

three experiments. Solutions prepared from all of the urines, both normal and experimental, yielded identical results. They all reduced Benedict's sugar reagent strongly, though the original

TABLE IV.  
*Uracil Nucleoside.*

Rabbit 4. Weight 1,950 gm. 1.5 gm. = 0.170 gm. of N injected intraperitoneally on the 4th day; 1.5 gm. = 0.170 gm. of N injected subcutaneously on the 12th day; 3 gm. of yeast nucleic acid injected subcutaneously on the 16th day.

Day.	Volume. cc.	Total N. mg.	Urea N.		Undetermined N. mg.	Extra total N. mg.	Extra urea N. mg.	Extra undeter- mined N. mg.	N in Hg pre- cipitate. mg.	Uracil. mg.
			mg.	per cent						
1	205	736	622	85	114					
2	180	564	438	78	126					
3	175	564	452	80	112					
4	180	730	579	79	151	166	134	34	23.9	29
5	235	549	425	77	124			7		
6	235	620	506	82	114	56	61			
7	230	658	541	82	117	94	96			
8	200	740	623	84	117	176	295			
9	150	578	464	80	114	14	19			
10	155	502	404	80	98					
11	175	609	497	82	112				5.77	0
12	140	693	520	75	173	130	67	65	42.2	79
13	200	631	487	77	144	68	34	36	7.91	±
14	195	584	477	82	107	21	24			
15	165	449	352	78	97					0
16	160*	605	426	70	179	42		71		0
17	190	978	812	83	166	415	359	58		0
18	180	432	344	80	88					
19	250	726	566	78	160	163	113	52		

\* Part of food not eaten.

13.2 mg. of N precipitated by neutral lead acetate on the 15th day and 17.1 mg. on the 17th day.

urines gave negative tests. Pentose tests were negative. Portions of the solutions were evaporated to dryness and tested for

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uracil, always with negative results. These experiments prove conclusively that the nucleoside had not been excreted unchanged.

A single experiment was carried out on man to study a possible formation of uric acid from uracil nucleoside. A diet low in purines was taken for several days and then 1.5 gm. of uracil nucleoside were ingested in three equal portions at intervals of 2 hours on 1 day. For the data see Table V. No increase in excretion of uric acid was observed. The elimination of creatinine which was also followed showed no significant variations.

TABLE V.  
*Uracil Nucleoside.*

Man. Weight 64 kilos. On the 4th day, 1.5 gm. taken in three equal portions at 7.30, 9.30, and 11.30 a.m.

Day.	Volume. cc.	Uric acid. gm.	Uric acid per day. gm.	Creatinine. gm.	Creatinine per day. gm.	Total N. gm.	Total N per day. gm.	Remarks.
1	870		0.574		1.56		11.85	
2	720		0.537		1.54		11.70	
3 Day.*	470	0.256		0.725		6.14		.
3 Night.*	355	0.210	0.466	0.815	1.54	5.80	11.94	.
4 Day.	320	0.203		0.764		5.03		No uracil.
4 Night.	475	0.211	0.414	0.870	1.63	6.00	11.03	" "
5 Day.	350	0.214		0.800		5.12		" "

\* Day period, 7.30 a.m. to 6 p.m.; night period, 6 p.m. to 7.30 a.m.

It may be concluded from these experiments that uracil nucleoside is destroyed in the body and not eliminated as such. The excretion of small quantities of uracil indicates that, at least in part, the uracil nucleoside is split to form pentose and uracil, and the resulting uracil is eliminated. The experiments of Mendel and Myers indicate that practically none of the uracil is destroyed in the body. Our relatively small recovery of uracil indicates that the destruction of uracil nucleoside in the body goes along two paths. In one case the pyrimidine is set free and is eliminated. In the other case the pyrimidine ring is changed before the sugar is split off so that it may be completely metabolized in the body. Presumably, the nitrogen is excreted as

urea but the urea output is increased so much, due apparently to increased protein metabolism, that an increase due to uracil nitrogen cannot be detected.

Five experiments with the nucleotide preparation were carried out on three different animals. The data are recorded in Tables

TABLE VI.  
Nucleotide.

Rabbit 5. Weight 2,500 gm. 3 gm. = 0.266 gm. of N fed *per os* on the 4th day; 2 gm. = 0.177 gm. of N injected intraperitoneally on the 11th day; food increased on the 8th day.

Day.	Volume. cc.	Total N. mg.	Urea N.		Extra total N. mg.	Extra urea N. mg.	Creatinine N. mg.	Undetermined N. mg.	Purine N mg.
			mg.	per cent					
1	250	572	433	76			33	106	
2	304	762	563	74			45	154	
3	244	617	458	74			34	125	7.5
4	232	674	533	79	24	49	35	106	
5	275	916	726	79	266	242	41	149	13.5
6	256	749	575	77	99	91	36	144	
7	250	722	553	77	72	69	34	135	
8	290	884	687	78			41	156	
9	233	812	627	77			33	152	
10	295	734	555	76			34	145	10.1
11	320	866	689	80	93	148	34	143	
12	250	644	460	71			35	149	
13	285	579	434	75			27	118	16.2

Traces of creatine were found on the 9th to the 12th days.

VI, VII, and VIII. As stated above, the preparation used was a mixture of uracil and cytosine nucleotide prepared from yeast nucleic acid. Analysis does not indicate the purity of this mixture and the quantity of uracil nucleotide cannot be accurately determined. Probably there was more uracil nucleotide present than cytosine nucleotide. Portions of 3 and 2 gm. were administered in different ways to rabbits and studied as in the experiments just described. The quantities of uracil given were not more than 50 to 60 per cent of those in the previous

## Pyrimidine Metabolism

experiment. The quantities administered were minimal on account of the tendency toward diarrhea produced by the compounds and the consequent interference with the absorption of food.

The urea nitrogen was increased on the days following the administration of the nucleotide preparations in all of the experiments. In most cases the increase was enough or more than enough to account for all of the nitrogen administered, in spite of the tendency toward diarrhea and possible decreased absorption of food. The creatinine and creatine excretions were unchanged in the one animal studied. The purine nitrogen showed insignificant increases during the experimental periods.

TABLE VII.  
Nucleotide.

Rabbit 6. Weight 1,960 gm. 2 gm. = 0.177 gm. of N fed *per os* on the 5th day.

Day.	Volume. cc.	Total N. mg.	Urea N.		Undetermined N. mg.	Extra total N. mg.	Extra urea N. mg.	N in Hg pre- cipitate. mg.	Uracil. mg.
			mg.	per cent					
1	225	495	382	77	113				
2	195	396	283	71	113				
3	175	517	406	78	111				
4	170	444	324	73	120			7.6	
5	170	633	511	80	122	170	162	25.2	19
6	175	493	393	80	100	30	44		
7	165	512	387	76	125	49	38		

The undetermined nitrogen was not increased after giving the nucleotide in three experiments, and the increase was only slight in the two others. This is in sharp contrast with the experiment using the nucleoside where the undetermined nitrogen was increased considerably. Nevertheless, some of the urines were examined for uracil, and it was recovered in all the experimental urines studied. The small quantities recovered did not permit suitable purification, but all specimens showed characteristic crystals and brilliant color reactions. In Experiment VIII

the uracil preparations were mixed, recrystallized once, and analyzed by micro Kjeldahl. 22 per cent of N was found instead of 25 per cent.

In the second part of Experiment VIII the neutral lead acetate and basic lead acetate fractions were analyzed for nitrogen to see if quantities of nucleotide and nucleoside were excreted.

TABLE VIII.  
*Nucleotide.*

Rabbit 7. Weight 2,150 gm. 3 gm. = 0.266 gm. of N injected intraperitoneally on the 5th day; 2 gm. = 0.177 gm. of N injected subcutaneously on the 12th day.

Day.	Volume. cc.	Total N. mg.	Urea N.		Undetermined N. mg.	Extra total N. mg.	Extra urea N. mg.	Extra undeter- mined N. mg.	N in Hg pre- cipitate. mg.	Uracil. mg.
			mg.	per cent						
1	150	657	541	82	116					
2	145	620	481	78	139					
3	140	513	412	80	101					
4	145	465	357	77	108					
5	100*	650	477	73	173	161	98	57	38.0	46
6	155	443	331	75	112					
7	130	462	348	75	114					
8	125	485	365	75	120					
9	145	511	412	81	99					
10	140	584	472	81	112					
11	150	555	462	83	98				2.3	0
12	110	777	606	78	171	207	139	69	12.7	13
13	185	594	478	80	116	24	11	14		
14	180	770	653	85	117	200	186	15		
15	150	625	515	82	110	55	48	8		
16	125	598	494	83	104	28	27	2		

\* Part of food not eaten.

On the 11th day there were 9.9 mg. of N precipitated by neutral lead acetate and 17.8 mg. precipitated by basic lead acetate. On the 12th day the values were 7.8 and 20.3 mg., respectively.

The uracil analysis yielded 21.8 per cent of N instead of 25 per cent of N.

While in each case the experimental day yielded a few milligrams more nitrogen in each fraction, it is evident that no appreciable quantities of nucleotide or nucleoside were excreted.

Owing to the smaller quantities of combined uracil fed in the nucleotide experiments the quantities of nitrogen excreted in the mercury fraction and the amounts of uracil recovered cannot be accurately compared with the similar quantities in the nucleoside experiments. But calculating roughly, it would seem that when either the nucleoside or nucleotide of uracil was administered, similar proportions of the compounds were decomposed completely in the body and similar proportions of the uracil were liberated and excreted uncombined.

A single experiment in which yeast nucleic acid was given is shown in Table IV. 3 gm. were injected subcutaneously, resulting in a large excretion of urea nitrogen on the 2nd day. There was a moderate increase in undetermined nitrogen, but no uracil could be detected in the mercury fraction on the 2 days following the injection. The basic lead acetate precipitates contained similar quantities of nitrogen indicating that no nucleotide or nucleoside was excreted. This experiment demonstrates that after the subcutaneous injection of yeast nucleic acid it is not excreted in the urine as such nor in the form of nucleotides or nucleosides. The absence of even traces of uracil confirms the observations of Mendel and Myers who fed yeast nucleic acid, and agrees with the results of Sweet and Levene who fed animal nucleic acid.

To summarize, it appears that when the pyrimidine, uracil, is taken into the body it is quantitatively excreted unchanged. But when uracil combined in the form of a nucleoside or nucleotide is administered only a small portion of the uracil is excreted. The uracil of yeast nucleic acid, however, seems to suffer complete destruction within the organism. Free thymine and the thymine combined in animal nucleic acid were found by Sweet and Levene to show the same relationships.

These data offer an insight into the intermediary metabolism of the pyrimidine fraction (at least the uracil fraction) of the nucleic acids. After the ingestion of nucleic acid the pyrimidines are destroyed in the body and their nitrogen excreted in the form of urea. The first step in metabolism is not a splitting off of the pyrimidine group from the nucleic acid but some change in the pyrimidine such as the splitting of the ring, even before the nucleic acid is broken up into its simple nucleotides and nucleosides.

Observations made with purine compounds offer a striking parallelism. Jones (7) and Amberg and Jones (8) have shown that deamination of purines may occur while they are still in combination. These observations together with the discovery by Benedict and his collaborators (9) of combined uric acid in blood, point to the probability that much of the metabolism of the purines may occur while they are still in combination.

Thus it appears that the intermediary metabolism of nucleic acids involves radical changes in both the purine and the pyrimidine groups before the relatively complex combinations are broken up.

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## STUDIES IN CREATINE AND CREATININE METABOLISM.

### V. THE METABOLISM OF CREATINE.

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In 1906 Folin (1) published a memorable contribution to the subject of creatine metabolism in which, as a result of experiments upon human subjects, he concluded that the animal organism cannot convert creatine into creatinine. The conclusion was also reached that while creatinine is a waste product, creatine is a food and that there is no biological relationship between these compounds. Klercker (2) reached quite similar conclusions independently of Folin's observations.

In view of the very recent admirable summary of the literature on creatine and creatinine given by Hunter (3), it is quite unnecessary for us to present an account of the mass of data which has accumulated since Folin's first contribution. A number of investigators have reported that traces of extra creatinine may appear in the urine following creatine administration and that with very large doses of creatine (Rose and Dimmitt, 4) appreciable quantities of extra creatinine may be eliminated. Such findings, together with the very excellent and suggestive work of Myers and Fine upon the relationship between muscle creatine and urinary creatinine elimination, have led many to question seriously Folin's original conclusions. Yet all the evidence presented on the origin of urinary creatinine from creatine during the 16 years which have elapsed since Folin's paper, has not been very convincing. Folin himself has never abandoned his original position, and there are many who would agree with him that practically no positive evidence has been offered that creatine is the normal biological source of creatinine. Even those most strongly favoring the view

that creatinine arises from creatine would have to admit that the evidence for this view must be selected piecemeal here and there, wherever it can be found. Direct, clear-cut evidence has been wholly lacking. The failure to find conversion of appreciable amounts of creatine into creatinine, together with the fact that "creatinuria" is a definite entity which is usually unrelated to changes in the creatinine elimination, are facts which have made Folin's position almost unassailable for those who forget the chemical relationship of the compounds involved.

The work reported in the present paper was a result of the conclusions reached by Behre and Benedict (5) that creatinine does not exist in appreciable amounts, if at all, in blood, while creatine (or some combination of it) can be demonstrated in this fluid, and accumulates in large amounts after ablation of the kidney function. Prior to these findings we had held the position taken by Folin, but the work on creatine and creatinine in blood forced us to a different view-point. We concluded that creatine, or some simple derivative of it, must be the precursor of creatinine, and therefore sought for loopholes in previous investigations which might open a way to attack the problem, so that some direct answer might be obtained to the fundamental questions involved. A careful consideration of all the facts available led us to conclude that there was one basic defect in the strongest argument advanced by Folin and his followers against the origin of urinary creatinine from creatine. Creatine was administered and did not increase the urinary creatinine. *But the creatine administered was never accounted for in any way whatever.* Here lay the weakness in Folin's argument. It is true that, reasoning from the known facts of metabolism, it seemed safe to assume that a nitrogenous compound would surely give rise to its end-products in the body during the period covered by Folin's experiments. Yet Folin himself demonstrated that creatine was practically or wholly unique among nitrogen compounds in that it failed to reappear in any form—even undetermined nitrogen—during the period of his observation. *Obviously, if metabolized at all, the creatine must ultimately give rise to something. Then so long as it remains wholly unaccounted for, there is no justification for the conclusion that it may not give rise to creatinine.*

As a result of these considerations we were led to undertake the experiments reported in the present paper.

The general plan of the work was to bring dogs into approximate nitrogenous equilibrium upon a constant diet, and then to administer a small amount of creatine daily until an appreciable quantity of the administered creatine was accounted for in some form in the urine. The results obtained are, we believe, of definite interest in connection with the basic questions of creatine and creatinine metabolism.

The work was carried out during the spring and summer of 1922. The experimental animals were healthy female dogs. Each was kept in a metabolism cage and fed upon a constant diet throughout the experiment. The animals were catheterized and weighed once daily before feeding. Periods were marked for the feces by administration of bone-black. Total nitrogen was determined by the macro Kjeldahl method, using potassium and copper sulfates as catalysts. Creatinine was determined by the original Folin method, using bichromate as standard. With very few exceptions the urines were diluted to the same volume each day and the reaction and colorimetric reading were thus carried out under rigidly fixed conditions. Creatine in the urine was converted to creatinine and determined as such by the procedure commonly employed in this laboratory (6). The creatine employed was prepared in the laboratory from creatinine obtained from urine (7) and was recrystallized until free from any detectable trace of creatinine.

The method for administration of the creatine was carefully considered. We finally adopted oral administration by mixing the creatine with the food as the method to be tried first. It seemed probable that the experimental period would be long, and the oral administration was far preferable from the practical standpoint to any injection procedure. It is time-saving, and avoids infection and chance of conversion of creatine to creatinine during sterilization of the solutions. We soon found that we could check up the question of conversion of creatine to creatinine in the gastrointestinal tract, as well as that of absorption of the creatine as such. As we shall show later in this paper, the oral method of administration is ideal from every standpoint for the experiments which we carried out upon dogs.

In each experiment the animal was placed upon the weighed diet, and became accustomed to the cage and routine of the experiments many days before the first periods which we have reported in the present paper.

The results of the experiments are recorded in Tables I to III. Table I presents a detailed report of our first experiment. Including the preliminary period of 9 days, this experiment lasted 128 days. There were ten periods of 7 days each, during which the dog received 0.620 gm. of creatine daily (containing 1 molecule of water of crystallization). This was equivalent to 0.470 gm. expressed as creatinine. The ten periods of creatine administration were followed by seven after periods of 7 days each.

We believe that a detailed study of Table I will be found of interest. We may summarize the main facts brought out as follows:

The dog had been on the diet for 24 days before the preliminary period reported. The nitrogen balance was + 1.3 gm. for a period of 7 days preceding the first period recorded in the table. The preliminary period reported shows a plus nitrogen balance of 0.7 gm. for 7 days. The creatinine output is quite constant for this (and the preceding unreported periods) and averages 405 mg. per day. During the 1st week of the creatine administration there is no apparent effect on the creatinine elimination, except that on the last day of the period an increase of about 20 mg. above the average level is to be noted. Such a slight increase is typical of results previously reported after creatine administration. The nitrogen balance of the first creatine period is +2.7 gm. This represents a retention of nitrogen equal to more than twice that contained in the creatine. None of the creatine given appears as such in the urine. Since the urinary nitrogen is definitely below that of the preliminary period we may infer that the creatine is practically wholly retained in some form during this period. The second period of creatine administration develops several points of real interest. The creatinine in the urine is definitely above that of the preceding period, but shows its maximal height during the 1st day. The creatinine for this second period averages about 40 mg. higher per day than is found during the control periods. This increase appears to us as still too small to warrant concluding that there is conversion of creatine into creatinine, but it begins to be very suggestive.

TABLE I.  
*Nitrogen, Creatinine, and Creatine Elimination after Creatine  
Administration.*

## Dog 1. Female.

The dog received daily the following diet:

Cracker meal.....	.....	gm.	150
Evaporated milk.....	.....	.....	100
Casein.....	.....	.....	24
(6.65 gm. nitrogen)			

Weight of dog. kg.	Daily output in the urine.			Total nitrogen for period. gm.	Remarks.
	Total nitrogen. gm.	Creatinine. mg.	Creatine (expressed as creatinin). mg.		
14.4	5.711	418			Preliminary period of 9 days duration. No creatine given.
	5.571	405			
	6.000	413	0	Urine = 50.686	
	5.600	405		Feces = 8.282	
	5.934	405		Hair = 0.115	
	5.700	397		Total = 59.083	
	5.460	405			
14.3	5.350	397		Food N = 59.850	
	5.360	401	0	Balance = +0.767	
	5.631	405	0		Average daily output.
14.4	5.740	405	0	Urine = 38.626	First period of creatine administration. 0.620 gm. of creatine ( $C_4H_8N_2O_2 \cdot H_2O$ ) was mixed with the food each day. This quantity of creatine would represent 0.470 gm. of creatinine.
	5.124	393	0	Feces = 6.316	
	5.418	397	0	Hair = 0.992	
	5.600	405	0	Total = 45.034	
	5.500	381		Food N = 46.55	
	5.644	405			
	5.600	426	0	Creatine N = 1.215	
				Total = 47.765	
				Balance = +2.731	
14.4	38.626	2,812	0		Total for 7 days.
	5.518	401	0		Average for 1 day.

## Creatine Metabolism

TABLE I—Continued.

Weight of dog. kg.	Daily output in the urine.				Total nitrogen for period. gm.	Remarks.
	Total nitrogen. gm.	Creatinine. mg.	Creatine (expressed as creatinine). mg.			
14.4	5.740	450	0	Intake	= 47.765	Second period of creatine administration. 0.620 gm. of creatine was given daily in the food.
	5.571	445	0	Output	= 44.036	
	5.380	450	32			
	5.030	445	83			
	5.210	450	113	Balance	= +3.729	
	5.270	445	130			
14.5	5.235	440	147			
	37.436	3,125	505			Total for 7 days.
14.4	5.348	446	101			Average for 1 day.
14.6	5.124	448	139	Intake	= 47.765	Third period of continued creatine administration.
	5.040	445	169	Output	= 42.049	
	5.207	450	164			
	5.151	460	163			
	5.040	450	203	Balance	= +5.716	
	5.124	455	209			
15.0	5.000	460	204			
	35.686	3,168	1,251			Total for 7 days.
14.7	5.098	452	178			Average for 1 day.
14.8	5.264	466	209	Intake	= 47.765	Fourth period of continued creatine administration.
	5.059	477	221	Output	= 42.252	
	5.235	471	239			
	5.291	477	253			
	5.040	466	245	Balance	= +5.513	
	5.012	482	254			
14.9	5.096	482	254			
	35.997	3,321	1,675			Total for 7 days.
14.8	5.142	474	239			Average for 1 day.

TABLE I—Continued.

Weight of dog. kg.	Daily output in the urine.				Remarks.
	Total nitrogen. gm.	Creatinine. mg.	Creatine (expressed as creatinine). mg.	Total nitrogen for period. gm.	
15.0	5.151	494	242	Intake = 47.765	Fifth period of continued creatinine administration.
	5.067	488	248		
	4.800	494	230	Output = 39.875	
	4.700	494	217		
	4.760	500	223	Balance = +7.890	
	4.550	494	204		
15.3	4.760	506	198		
	33.788	3,470	1,562		Total for 7 days.
15.1	4.826	495	223		Average for 1 day.
15.3	5.040	506	224	Intake = 47.765	Sixth period of continued creatinine administration.
	5.040	526	230		
	4.760	513	258	Output = 40.054	
	4.731	494	242		
	4.788	506	218	Balance = +7.711	
	4.861	488	229		
15.4	4.760	500	204		
	33.980	3,533	1,605		Total for 7 days.
15.3	4.854	504	229		Average for 1 day.
15.4	4.900	519	198	Intake = 47.765	Seventh period of con- tinued creatine admin- istration.
	4.927	506	192		
	4.970	500	186	Output = 41.017	
	4.956	506	211		
	5.166	526	210	Balance = +6.748	
	5.151	513	237		
15.5	5.040	526	245		
	35.110	3,596	1,479		Total for 7 days.
15.5	5.015	513	211		Average for 1 day.

## Creatine Metabolism

TABLE I—Continued.

Weight of dog. kg.	Daily output in the urine.				Remarks.
	Total nitrogen. gm.	Creatinine: mg.	Creatine (expressed as creatinine). mg.	Total nitrogen for period. gm.	
15.8	5.012	506	205	Intake = 47.765	Eighth period of continued creatine administration.
	5.026	500	187	Output = 44.296	
15.0	5.375	540	203	Balance = +3.469	
	5.823	560	192		
	5.754	555	224		
	5.684	533	285		
15.5	5.544	540	286		
	38.218	3,784	1,582		Total for 7 days.
15.5	5.459	533	226		Average for 1 day.
15.7	5.375	526	284	Intake = 47.765	Ninth period of continued creatine administration.
	5.081	519	253	Output = 41.771	
	5.124	533	238	Balance = +5.994	
	5.070	526	224		
	5.320	526	253		
	5.067	533	231		
15.7	4.760	519	205		
	35.797	3,682	1,688		Total for 7 days.
15.7	5.113	526	241		Average for 1 day.
15.7	5.012	526	210	Intake = 47.765	Tenth period of continued creatine administration.
	4.927	526	210	Output = 42.310	
	5.110	540	224	Balance = +5.455	
	5.067	570	201		
	5.460	555	294		
	5.290	540	270		
15.6	5.194	533	238		
	36.060	3,790	1,647		Total for 7 days.
15.7	5.151	541	235		Average for 1 day.

TABLE I—Continued.

Weight of dog. kg.	Daily output in the urine.					Remarks.
	Total nitrogen. gm.	Creatinine. mg.	Creatine (expressed as creatinine). mg.		Total nitrogen for period. gm.	
15.7	5.250	519	36	Intake	= 46.550	First after period. No creatinine given.
	5.012	513	0	Output	= 42.745	
	5.290	570	0	Balance	= +3.805	
	5.375	555	0			
	5.320	540	0			
	5.110	506	0			
15.6	5.460	506	0			
15.7	36.817	3,709	36			Total for 7 days. Average for 1 day.
	5.259	529				
15.6	5.264	494	0	Intake	= 46.550	Second after period. No creatinine given.
	4.703	482	0	Output	= 41.370	
	4.941	488	0	Balance	= +5.18	
	4.816	500				
	4.563	488				
	5.460	494				
	5.193	506				
15.6	34.940	3,452				Total for 7 days. Average for 1 day.
	4.991	493				
15.7	5.040	500		Intake	= 46.550	Third after period. No creatinine given.
	5.123	506		Output	= 42.375	
	4.983	494	0	Balance	= +4.175	
	4.900	488				
	5.096	482	0			
	5.488	500				
15.7	5.180	494	0			
15.7	35.810	3,464	0			Total for 7 days. Average for 1 day.
	5.115	494				

TABLE I—Concluded.

Weight of dog. <i>kg.</i>	Daily output in the urine.				Remarks.
	Total nitrogen. <i>gm.</i>	Creatinine. <i>mg.</i>	Creatine (expressed as creatinine). <i>mg.</i>	Total nitrogen for period. <i>gm.</i>	
15.7	5.290	506	0	Intake = 46.550	Fourth after period. No creatine given.
	5.473	466		Output = 44.340	
	5.576	486		Balance = +2.210	
	5.334	455		.	
	5.410	463		.	
	5.320	450		.	
	5.221	440		.	
15.7	37.624	3,266			Total for 7 days.
	5.374	466			Average for 1 day.
15.7	5.100	441	0	Intake = 39.900	Fifth after period. No creatine given.
	5.040	455		Output = 37.196	
	5.207	468		Balance = +2.704	
	5.320	463		.	
	4.983	440		.	
	5.070	455		.	
	30.720	2,722		.	
15.7	5.120	453			Total for 6 days.
					Average for 1 day.
15.8	5.040	440	0	Intake = 46.550	Sixth after period. No creatine given.
	5.400	450		Output = 43.965	
	5.375	440		Balance = +2.585	
	5.490	440		.	
	5.515	445		.	
	5.291	445		.	
	5.390	440		.	
15.8	37.501	3,100			Total for 7 days.
	5.357	442			
15.8	5.235	435	0	Intake = 46.550	Seventh after period. No creatine given.
	5.264	440		Output = 43.910	
	5.124	435		Balance = +2.640	
	5.320	435		.	
	5.242	442		.	
	5.291	426		.	
	5.348	426		.	
15.8	36.824	3,039			Total for 7 days.
	5.260	434			
					Average for 1 day.

On the 3rd day of this period, or the 10th day of creatine administration, a scarcely detectable quantity of creatine (32 mg.)<sup>1</sup> appears in the urine. The creatine in the urine rapidly increases, and within 5 days has reached 147 mg. for 1 day. Here is direct evidence that creatine given even in small amounts, and apparently completely utilized, is retained, at least partially, as such in the organism for a considerable period. Furthermore, many days are required to fill the creatine reservoir, indicating a very slow rate of metabolism for creatine. The nitrogen balance for the second period of creatine feeding is +3.7 gm., representing a storage of nitrogen of more than three times the quantity contained in the creatine retained. This result begins to suggest that creatine may cause nitrogen storage in the body far beyond that contained in the creatine itself. Further data show that there can be little question of the correctness of this view.

The third period of creatine administration (Table I) throws further light upon the questions raised. The creatinine output rises. True, it rises grudgingly and unwillingly, with frequent minor drops. This picture is typical of all our experiments in this connection. But the period as a whole shows a definite increase in creatinine excretion. The figure is now about 50 mg. per day (12 per cent) above the control period, and we should almost feel justified in concluding definitely that creatine gives rise to creatinine in the organism, but with the proviso that this is apparently not a simple direct conversion. Definite settlement of this question will come later. The creatine output in the urine continues to rise, reaching about 200 mg. per day toward the end of the third period of creatine administration. Evidently the creatine reservoir can retain less and less of the ingested creatine, but more than 50 per cent of that given is still retained. The nitrogen balance for this period is +5.7 gm., and the weight of the dog is definitely increasing. We are inclined to believe that the nitrogen retention and increase in weight are due to the creatine administered, since during the 33 days before creatine administration this animal did not change in weight a total of more than 100 gm., and the fluctuations were both sides of 14.4 kilos.

<sup>1</sup> Throughout this paper the creatine found in the urine is expressed as its equivalent in creatinine.

During the first 21 days of the creatine feeding the weight of the animal has increased 600 gm. The finding is at least very suggestive.

During the fourth period of creatine administration the creatinine in the urine shows the same continued increase as for the two preceding periods. The average for the period shows a daily elimination of 70 mg. of creatinine (17 per cent) above the daily average for the control periods. The urinary creatine continues to rise, and shows a daily average of 239 mg. This figure is nearly maximal for the entire ten periods of creatine administration, and is maintained approximately constant as long as the creatine feeding is continued. The creatine eliminated during these periods amounts to 50 per cent of that given.

In spite of the increased creatinine and creatine in the urine, the total urinary nitrogen continues low and the nitrogen balance for the period is +5.5 gm. The weight of the dog shows a slight increase (100 gm.) over the preceding period.

The picture for the fifth and sixth periods of creatine feeding is similar to that of the preceding period. Creatinine in the urine continues to increase slowly, so that the sixth period shows 100 mg. of creatinine per day in the urine in excess of that for the control periods. The daily output of creatine is somewhat irregular, but the average for the fifth and sixth periods is close to the average for the fourth period. Retention of nitrogen is over 1 gm. per day for the two periods, and the weight of the animal increases by about 400 gm.

The seventh period of creatine feeding shows a slight further rise in creatinine output, while the creatine output is slightly lower than for the preceding period. Nitrogen retention for the 7 days is 6.7 gm. The average weight of the animal for this period is 1,100 gm. greater than at the beginning of the creatine administration.

The eighth and ninth periods of creatine feeding show continued increase in the creatinine elimination, reaching a daily average of 121 mg. (29 per cent) in excess of the control period. The creatine output is practically constant. Nitrogen retention for the two periods is 9.3 gm. and the weight of the dog reaches 1,300 gm. above that of the control period.

The tenth and last period of creatine administration shows wider fluctuations in the creatinine elimination than do the preceding periods. The maximal figure for creatinine elimination is reached during this period, the average daily output being 541 mg., which is 136 mg. or 33 per cent above the daily output (405 mg.) during the control period. The highest figure for a single day (570 mg.) occurs once during this and the following period. The relatively wide variations which seem to occur at the height of the curve of creatinine elimination (Period 10 and the first after period) are not apparent during the subsequent periods. The creatine output for the tenth period is similar to that for the ninth.

At the end of the tenth period of creatine administration the feeding of creatine was discontinued. We watched the figures for the following period with exceptional interest. If the increased creatinine, which we had found in the urine, represented creatinine formed in the intestinal tract through bacterial or other action, then the excess creatinine in the urine should vanish as promptly, or even more promptly, than the creatine, after the latter was discontinued. If, on the contrary, the extra creatinine in the urine represented the result of a slow, definite metabolism of the previously administered creatine, the withdrawal of the creatine should have an effect which should show itself only slowly over a considerable period of time.

The results for the first and subsequent after periods are beautiful and clear-cut. The first day after withdrawal of the creatine from the food the creatine of the urine fell to a scarcely detectable quantity (36 mg.) and at no subsequent time during the seven after periods could we detect any creatine in the urine. The creatinine of the urine, on the contrary, appears at first to have no real relationship to the administered creatine. The independence of creatine and creatinine is again apparent. But this independence is only apparent; it is not real. Beginning with the first after period, and continuing through the six following periods, there is a drop in the creatinine excretion which becomes perfectly plain and definite when we consider the figures for any 7 day period. The drop is just as unwilling, slow, and slightly irregular (from day to day) as was the rise. But it is just as definite and clear-cut for a number of days considered together. For the fifth after period the creatinine coefficient (28) is the same as for

the preliminary period. The dog continues to store nitrogen, but this falls to 2.2 and 2.7 gm. for the last two periods of observation. After discontinuing the creatine the weight of the animal remains practically constant at an average of 15.75 kilos for the seven after periods as against 15.7 kilos for the first after period.

Tables II and III record two further experiments similar to that recorded in Table I. In order to save space the figures are given for 7 day periods, and for the daily average of each period, instead of in full detail as for the experiment recorded in Table I.

The experiment with Dog 2 is summarized in Table II. The entire experimental period covered twelve periods of 7 days each. These included two preliminary control periods, five periods of creatine administration, and five after periods. 0.5 gm. of creatine was given daily with the food during the creatine periods, equivalent to 379 mg. per day expressed as creatinine. This represented 43 mg. per kilo of body weight, which is the same per kilo dose as that given to Dog 1. There is a slight but detectable rise in creatinine elimination for the first creatine period, while the maximal figure for creatinine in the urine was reached for the third creatine period. For this period the creatinine output per day was 387 mg. This was 92 mg., or 31 per cent, above the preliminary period level of 295 mg. of creatinine per day. This high creatinine elimination was approximately maintained through the last two periods of the creatine administration. After withdrawal of the creatine from the food the creatinine shows the same type of very slow fall observed in the experiment with Dog 1 (Table I).

In the experiment with Dog 2 creatine (40 mg.) appeared in the urine first on the 22nd day of creatine administration, or 11 days later than in the case of Dog 1, though both received the same quantity of creatine per kilo of body weight. The quantity of creatine in the urine increased rapidly just as in the preceding experiment, and during the fifth (and last) period of creatine administration represented about 50 per cent of that given, just as in the preceding experiment. Creatine (25 mg.) appeared for the last time in the urine on the 1st day of the first after period. Nitrogen retention and increase in weight of the animal occurs, but these are not so great as in the case of Dog 1. Nevertheless, there is a considerable excess of nitrogen (above that contained in

the creatine) retained in this animal, and there is a slight increase in weight. The period of creatine administration was only one-half as long as in the preceding experiment.

TABLE II.  
*Nitrogen, Creatinine, and Creatine Elimination after Creatine Administration.*  
Dog 2. Female.

The dog received daily the following diet:

	gm.
Cracker meal.....	150
Evaporated milk.....	100
Casein.....	15
Bone ash.....	40

(5.45 gm. nitrogen)

The figures are for 7 day periods unless otherwise indicated.

Average weight of dog. kg.	Total nitrogen. gm.	Creatinine. mg.	Creatine (expressed as creatinine). mg.	Average creatinine for 1 day. mg.	Nitrogen balance. gm.	Remarks.		
11.6	31.774	2,060	0	295	+0.240	First preliminary period.		
11.6	31.916	2,075	0	295	+0.491	Second preliminary period.		
11.6	31.650	2,197	0	314	+1.246	Dog received 0.5 gm. creatine (C <sub>4</sub> H <sub>9</sub> N <sub>3</sub> O <sub>2</sub> ·H <sub>2</sub> O) daily with the food.		
11.7	31.938	2,507	0	358	+1.293	Creatine given as in preceding period.		
11.8	31.777	2,725	0	387	+1.236	"	"	"
11.8	32.222	2,623	837	374	+0.909	"	"	"
11.9	31.833	2,610	1,025	374	+0.840	"	"	"
12.1	31.398	2,545	25*	363	+0.628	No creatine given.		
12.2	30.752	2,487	0	355	+1.336	"	"	"
12.2	30.981	2,403	0	343	+1.149	"	"	"
12.3	31.743	2,234	0	319	+0.237	"	"	"
12.3	32.056	2,209	0	315	-0.029	"	"	"

\* Eliminated 1st day of period.

In Table III is recorded a summary of our third experiment, carried out upon Dog 3. The experiment covers a period of 92 days. 450 mg. of creatine, equivalent to 341 mg. expressed as

## Creatine Metabolism

creatinine, were given daily during the period of creatine feeding, which lasted 66 days. There was an after period of 20 days. The

TABLE III.

*Nitrogen, Creatinine, and Creatine Elimination after Creatine Administration.*

Dog 3. Female.

The dog received daily the following diet:

		gm.
Cracker meal.....		90
Evaporated milk.....		100
Casein.....		15
(4.45 gm. nitrogen)		

The figures are for 7 day periods unless otherwise indicated.

Average weight of dog. kg.	Total nitrogen. gm.	Creatinine. mg.	Creatine (expressed as creatinine). mg.	Average creatinine for 1 day. mg.	Nitrogen balance. gm.	Remarks.		
9.1	22.188	1,662	0	277	+0.300	6 day preliminary period.		
9.1	24.052	2,039	27*	291	+2.092	Dog received 0.450 gm. creatine ( $C_4H_9N_3O_2 \cdot H_2O$ ) daily with the food.		
9.1	25.640	2,224	1,092	317	+1.650	Creatine given as in preceding period.		
9.2	24.881	2,277	1,288	325	+2.361	" " "		
9.2	24.602	2,298	1,189	328	+2.723	" " "		
9.1	23.739	2,344	1,371	335	+3.667	" " "		
9.2	23.630	2,301	1,324	328	+3.689	" " "		
9.2	23.731	2,347	1,270	335	+3.520	" " "		
9.2	24.121	2,367	1,204	338	+3.145	" " "		
9.3	23.283	2,364	1,150	337	+3.574	" " "		
9.2	9.980	1,001	510	333	+1.584	3 day period. Creatine given as in preceding period.		
9.2	19.517	1,984	20†	330	+2.745	6 day period. No creatine given.		
9.1	24.266	2,181	0	311	+2.058	No creatine given.		
9.0	25.474	2,058	0	294	+0.281	" " "		

\* Eliminated last day of period.

† Eliminated 1st day of period.

results of the experiment are quite similar to those of the preceding experiments. The creatinine output shows a slight rise during the first creatine period. The rise continues, with slight fluctuations, and the creatinine output reaches a maximal figure of 338 mg. per day for the eighth period of creatine administration. This figure is 61 mg., or 22 per cent, above the control period. Creatine first appeared in the urine on the 7th day of creatine feeding, and increased rapidly as in the preceding experiments. When the creatine output was at its height, about 54 per cent of the creatine given was excreted in the urine as such. When the creatine feeding is stopped, creatine disappears from the urine promptly, and the urinary creatinine shows the slow but steady fall noted in the previous experiments. The figures for the nitrogen balance show that there is unquestionably an excess storage of nitrogen in the organism associated with the creatine administration. The weight of this animal increased very little during the period of creatine feeding, but showed a definite decline after the creatine was stopped.

#### DISCUSSION.

The experiments recorded in the present paper seem to us to establish definitely the origin of urinary creatinine from creatine. But they also bring out some additional points of interest. Folin's view of the biological independence of creatine and creatinine is shown to be incorrect. However, our work demonstrates that the metabolism of creatine in the animal body is unique in many respects. *Direct* conversion of creatine into creatinine is apparently not a normal process in the animal organism. One or more intermediate reactions are probably involved. The quantity of creatine which can be metabolized in the organism is closely limited, and the quantity of creatinine which can be formed is also restricted. The metabolism of creatine in the organism is, unlike that of other known nitrogenous compounds, very slow, requiring days or more probably weeks for its completion. We know of no parallel to this finding in the whole field of metabolism with the possible exception of thyroxin, which seems to act over a long period of time in the organism.

Our experiments seem to demonstrate almost conclusively that creatinine is but one of the end-products of creatine metabolism.<sup>2</sup> We may secure data from our experiments concerning the quantity of creatinine produced in the organism of the dog from a given amount of creatine by comparing the total creatine retained (*i.e.*, not eliminated as such) in our animals, with the quantity of creatinine eliminated in the urine in excess of that expected, for all the periods of observation. In making these calculations we have based the expected creatinine on that eliminated during the control periods in Dog 3, in which there was no appreciable change in weight. In the case of Dogs 1 and 2, in which there was an increase in weight during the experiments, we have calculated the expected creatinine on the basis of the creatinine coefficient for these animals during the control periods. This has seemed to us preferable to using the absolute creatinine output for the control periods in these animals, though the difference in the results would not be great, whichever basis of calculation is used.

We have summarized the results of these calculations in Table IV.

It will be seen from Table IV that approximately 30 per cent of the creatine actually retained by each dog was eliminated as creatinine. Since in each experiment the period of observation extended until the creatinine output had approximately reached

<sup>2</sup> This conclusion is similar to the one we reached some years ago as a result of our work on creatine excretion in phlorhizinized dogs (Benedict, S. R., and Osterberg, O., *J. Biol. Chem.*, 1914, xviii, 195). Hunter (3) has criticized our conclusions from that work upon the basis that our results "demonstrate at the utmost nothing more than the production, independently of tissue catabolism, of notable amounts of creatin in *phlorhizinized animals*. To infer a similar extensive production in normal animals is hardly justifiable." Hunter then goes on to say: "The production in phlorhizin poisoning of excessive amounts of sugar from protein does not prove that the metabolism of amino acids in the normal organism necessarily involves the intermediate production of glucose." We disagree with Hunter in regard to both of his statements above quoted. Reference to the work of Lusk, Dakin, and many other investigators who have studied glucose formation from amino-acids will show that these investigators have consistently drawn conclusions for the normal animal concerning the usual course of metabolism on the basis of results obtained in phlorhizin diabetes. (*cf.* Lusk, G., *The elements of the science of nutrition*, Philadelphia and London, 3rd edition, 1917.)

the base line, we may tentatively conclude that almost exactly one-third of the creatine metabolized was converted into creatinine. It may be urged that since we have left about 66 per cent of the retained creatine unaccounted for, this creatine may also ultimately reappear as creatinine in the urine. There is perhaps some justification for this view, but we feel that taking all the facts into account, the assumption is most reasonable that in our experiments we secured practically all of the creatinine which would appear from the ingested creatine. Thus in the case of Dog 2, where the after period extended over as long a time as the period of creatine ingestion, we find the creatinine coefficient

TABLE IV.

*Showing the Extra Urinary Creatinine Eliminated in Comparison with Creatine Retained\* for the Creatine Periods and After Periods of the Experiments Reported in Tables I, II, and III.*

Figures for creatine are expressed as creatinine.

Dog No.	Total creatine given.	Total creatine excreted as such.	Total creatine retained.	Total creatinine eliminated.	Total creatinine expected.	Extra creatinine eliminated.	Percentage of retained creatine eliminated as creatinine.
	gm.	gm.	gm.	gm.	gm.	gm.	per cent
1	32.9	13.0	19.9	56.96	51.16	5.80	29.1
2	13.2	1.9	11.3	24.54	20.65	3.89	34.2
3	22.5	10.44	12.06	27.78	23.82	3.96	32.7

\* Creatine not eliminated as such is regarded as retained for the purpose of this table.

fully down to that of the control period, and the drop in creatinine excretion for the last period quite negligible—averaging only 4 mg. per day. In this experiment the highest percentage (34.2) of creatine was accounted for as creatinine. We thus feel that in view of these facts, and the relatively close agreement for the percentage of creatine recovered as creatinine, for experiments which differed so widely in total duration, we may properly assume that practically all the creatinine which will arise from creatine was obtained in our experiments. It would thus appear that in the metabolism of creatine under the conditions of our experiments, from every 3 molecules of creatine metabolized, only 1 gives rise to creatinine. If indeed the remaining creatine yields

creatinine, then the course of this latter fraction would still appear to be different from that of the portion which yielded creatinine in our experiments. We feel warranted in tentatively concluding that the metabolism of creatine takes place through two or more different paths, only one of which yields creatinine.

If the above view-point, which we feel is demonstrated at least from a general standpoint, is adopted, *viz.* that only a portion of creatine metabolized yields creatinine, and this indirectly, we believe that some of the most fundamental difficulties in interpreting creatine and creatinine metabolism will disappear. The fact that creatinuria comes and goes quite independently of changes in the creatinine elimination can be understood on the basis that the fraction of creatine excreted during the creatinuria would never have yielded creatinine in the organism in any event. According to this view-point, there should be two distinct types of creatinuria. One would be similar to that of our experimental animals after continued ingestion of small amounts of creatine. Such creatinuria would result from an excess of creatine in the organism—either taken from without or synthesized by the tissues. The creatinurias of poor nutritive conditions would not be of this type for our experiments show that excess of free creatine in the normal organism results in a definitely increased creatinine output, and the creatinine output is usually neither above nor below normal in creatinuria. Thus increased creatinine output does not precede or accompany the creatinuria of inanition, etc. We believe that the defect in these conditions is associated with a failure in the utilization of a portion or all of that fraction (probably two-thirds) of creatine which normally never yields creatinine. Our experiments seem to indicate that the utilization of at least a part of the creatine is associated with anabolic processes which are at a minimum in the conditions ordinarily leading to creatinuria in the adult man or dog.

The creatinuria of infancy and childhood, on the contrary, is probably due (as has been suggested by others) to the presence of a real flood of synthetic creatine in the organism. Here the creatinine output increases with growth. Furthermore, the figures for nitrogen balances which we obtained seem to indicate that an excess of creatine would be desirable or necessary for the anabolic processes which are in the ascendant in the growing organism.

Creatininuria has been reported by some investigators as a result of forced protein feeding. We are inclined to question whether this form of creatininuria has any real significance in connection with creatine metabolism. We should tend to regard it as representing a forcing out of small quantities of creatine from the tissues by the tremendous influx of other nitrogenous compounds. The increased uric acid output following very excessive protein intake probably has a similar origin.

Are we to regard the creatine fraction which yields creatinine as of no significance in the organism? Does this represent merely a means of ridding the system of waste creatine? Is there no intermediary reaction of importance to the organism involved here? Our data would seem to indicate that the process in which creatinine is formed from creatine may indeed be of definite significance in the organism. The facts in favor of this view are: (1) The finding that the formation of creatinine from creatine is a definitely limited process—even with the most prolonged administration of creatine (70 days) we can increase the average daily output of creatinine only about 30 per cent. If creatine were directly converted to creatinine as an "exogenous" process, this conversion should not be so limited. (2) The slow increase which occurs in the creatinine output would seem to indicate that creatinine is produced only as creatine is metabolized through some slow and probably complex course in the body. (3) The almost absolute constancy of the creatinine output first demonstrated by Folin, and which holds under widely varying physiological and pathological conditions, would indicate the correctness of Folin's original view that creatinine indicates a specific form of metabolism of fundamental importance to living tissue. Creatine metabolism and creatinine formation surely stand apart from other known processes of metabolism. Creatinine formation must remain as the most strictly endogenous form of metabolism so far known. Yet under suitable conditions it is possible to raise this form of metabolism by about 30 per cent. This represents, we believe, approximately the maximal increase which can be produced in the normal adult organism. Folin's absolute line between endogenous and exogenous metabolism is here removed.

The question must remain open as to whether creatinine formation is a process essentially confined to muscular tissue (Shaffer), or whether it represents, as Folin believes "an index of the total

normal tissue metabolism." The general occurrence of creatine in the tissues of the body, together with its relatively high concentration in non-muscular tissue such as brain, leads us to favor Folin's view. In connection with Shaffer's demonstration of relationship between the creatinine coefficient and muscular development, it should be remembered that since muscular tissue represents so large a proportion of living tissue in the body, such a relationship would probably obtain whether creatinine production were confined to the muscles or not. Quite a similar relationship may exist between muscle mass and the various nitrogenous metabolites.

The parallelism between total body creatine and urinary creatinine demonstrated so convincingly by Myers and Fine, finds confirmation and explanation in the results which we have reported.

If the course of metabolic change of creatine formed in the body is as slow as we have shown it to be for creatine taken in from without (and there is no reason to assume the contrary), then the quantitative relationship of urinary creatinine to total nitrogen during a high calory, low protein diet demonstrated by Folin in 1905, may lose some of its significance as representing the real proportion of endogenous nitrogen which goes to creatinine. The creatinine in the urine may, according to our results, represent a portion of the creatine stored in the tissue weeks beforehand.

We have no data to offer at present concerning the mechanism of the storage of creatine in the animal body. The view of Folin and Denis (well discussed by Hunter (3)) that creatine in the muscle is in a complex combination, appears to us to be attractive, but to have no experimental evidence in its favor. Folin and Denis inferred a complex combination of creatine in the muscle because creatine would pass from the blood to the muscle, although this latter tissue showed a much higher concentration of creatine than did the blood. As Hunter very properly points out, such evidence could be just as well advanced in favor of a combination of the non-protein amino-acid content of muscle. It would apply also to potassium salts, etc. But on the other hand, the ease of extraction of creatine from muscle is not a cogent argument against its existence in a complex in the tissues. It would simply show that the combination was soluble. The boiling and hy-

drolysis always employed in the separation or determination of creatine in muscle might suffice to break up a creatine complex did such exist. Our results might lead one to infer the existence in the organism of some complex derived from creatine, though we have no indication that the creatine molecule would be essentially intact in such a complex. The peculiar function of the creatine in the body must remain an unsolved question for the present.

Our results seem to indicate the probable futility of such experiments as have been heretofore conducted to determine the specific precursors of creatine in the animal body. If the production of creatine in the organism is facultative, and does not exceed the creatine required by the normal tissues, such researches may be well nigh hopeless. If, on the contrary, creatine production can be increased by increase in the creatine precursor, then long continual experiments along this line may be successful.

In conclusion, it may be stated that we fully recognize that our quantitative interpretations may be subject to revision. Different species may show quite different quantitative relationships, and any given species may vary in its quantitative handling of creatine with different conditions of diet or of general nutrition. Our own experiments show a wide variation in the time required to induce creatininuria in different animals by limited creatine administration. But we feel that there can be no reasonable question of the correctness of our fundamental interpretations and the quantitative relationships appear to be clear-cut under the conditions of our experiments.

The work we have reported obviously leads to questions which should be settled promptly. We already have experiments well under way, by means of which we are studying various phases of the problems involved. We shall soon be able to report upon the quantity of creatine stored intact in the muscular tissue as a result of prolonged creatine ingestion. We shall also shortly be able to answer the question as to the maximal creatine retention obtainable with successively increased doses of creatine. Elimination of some creatine at a given level of creatine intake does not justify the conclusion that raising the level of the creatine intake will not result in increased creatine retention.

We are also investigating the question raised by the marked nitrogen retention and the tendency to increase in weight, which we have found in each of our experiments. Creatine appears to have a definite influence as a unique type of food. In this connection we are already investigating the effect of creatine upon the nutrition and growth of rats and pigeons, and are including in this study the question of whether an increase in the lipoid nitrogen follows prolonged creatine ingestion.

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## AQUEOUS EXTRACTS OF PANCREAS.

### I. INFLUENCE ON THE CARBOHYDRATE METABOLISM OF DEPANCREATIZED ANIMALS.

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#### INTRODUCTION.

The following study is a continuation of the investigation begun by Murlin and Kramer (1) in 1912 at the Cornell University Medical College and prosecuted by them at that place until late in August, 1916. The early results of that investigation showed a complete disappearance of sugar from the urine of depancreatized dogs when a combined extract of pancreas and duodenal mucosa was injected intravenously. Because of a remarkable coincidence the effect was erroneously attributed at the time to the influence of alkali administered with the extracts. Later results (2) proved, however, that alkali alone (sodium carbonate or bicarbonate) never caused complete disappearance of sugar from the urine and did not bring about either deposition of glycogen (3) or combustion of sugar in the depancreatized dog. This conclusion left the earlier result open to the interpretation that there had been an actual utilization of carbohydrate under the influence of the pancreatic extract, and it was to test this hypothesis that in 1916 extracts of pancreas alone, made in acid media, but administered with alkali and sugar by stomach tube, were used. In order to avoid any possible liberation of CO<sub>2</sub> from the alkali, which would vitiate the respiratory quotient as an index of combustion, sodium hydroxide in 0.05 N concentration was chosen. Encouraged by the results of Kleiner and Meltzer (4) with raw pancreatic material and because the extracts were not in these trials given parenterally, they were not boiled, as they had been in the earlier experiments. Following this proce-

dure (3) there was with two dogs in four trials a rise of the respiratory quotient of 10 to 18 points above the predetermined diabetic level, indicating a restoration in part of the power to oxidize glucose.

The work was unavoidably interrupted at this point, but was resumed by the senior author in October, 1921, with the conviction that assiduous search would disclose a method of extraction and purification of the antidiabetic substance (*insuline*, Schafer (5)) produced by the pancreas in a form capable of administration to diabetic *human* subjects. The starting point was the method of extraction employed by Murlin and Kramer in 1913; namely, maceration of the freshly removed pancreas in 0.2 N HCl and neutralization of the extract before administration. It was known from previous experience that such extracts not purified further are somewhat toxic. But it was believed that the toxic substance could be removed once the presence of the antidiabetic substance was extracted in sufficient amount to have significant therapeutic value. The method of purification in mind and subsequently employed was that used by Zuelzer (6) and his colleagues in 1908 and adopted by Collip (7) in purifying Banting's extract for administration to human beings; namely, precipitation of extraneous proteins by means of alcohol. Zuelzer unquestionably had a potent extract, but he did not succeed in removing all the toxic substances, and in the letters patent (8) granted by the U. S. Patent Office in 1912 admission is made that the extract was "too septic to be used for persons." Nevertheless, both Zuelzer (6) and Forschbach (9) had demonstrated the presence of an antidiabetic substance both experimentally on dogs and clinically. The efforts of Blumenthal (10) and of Croftan (11), who as far as we are aware was the first to act specifically upon the hypothesis that the hormone was destroyed by trypsin,<sup>1</sup> were also distinctly encouraging as was, of course, the more recent success of Kleiner (13) in reducing the blood sugar of depancreatized dogs by means of suspensions in sterile water of macerated fresh pancreas of the dog.

<sup>1</sup> The use of heat for the purpose of destroying the external enzymes had been used earlier by Leschke (12), but he was acting on just the opposite theory from Croftan; namely, that such effects as one gets from extract of pancreas on carbohydrate metabolism are due to the external enzymes themselves.

The negative results obtained by Hugounenq and Doyon (14) and by Hédon (15), of course, proved only that the particular methods employed by them had not successfully brought out or successfully preserved the hormone which everybody since Minkowski's time has known must be present. The detoxication theory was wholly incredible in the light of Hédon's (16) and Drennan's (17) work on transfusion of blood from the vein of a normal animal into a diabetic animal.

The idea of excluding the external enzymes by ligation of the pancreatic ducts and consequent degeneration of the acinous tissue was acted upon first by Scott (18) working at that time under Carlson, but succeeded by better surgical skill in the hands of Banting and Best (19). It should be emphasized that this technique was not necessary to demonstrate the presence of the hormone as Banting and his colleagues themselves have shown. The use of 0.2 N HCl in extraction of the pancreas by Kramer and Murlin, instead of just a sufficient amount of 0.1 N HCl to render the mixture acid as used by Knowlton and Starling (20), was begun with the idea of destroying trypsin,<sup>2</sup> because they had witnessed the terrific effects of surviving trypsin when extracts in neutral solutions were injected subcutaneously.

The immediate stimulus for resumption of this work was the report of Paulesco's (21) favorable results. They were distinctly encouraging. He found that the intravenous injection of a sterile extract into depancreatized dogs brought about a diminution or even a temporary suspension of the hyperglycemia and of the glycosuria; also a diminution of the excessive production and excretion of urea and acetone bodies. The effect appeared immediately, reached its maximum in about 2 hours, and continued for 12 hours. The method of extraction (22) employed by Paulesco has not been available to us.

#### *Experiments Previous to Appearance of Banting and Best's Method.*

In the process of reorientation after several years intermission, and before the perfusion method was fully proved out, simple

<sup>2</sup> The original idea of facilitating solution of the hormone from the pancreas by means of acid seems to have been adopted by Knowlton and Starling because secretin, the prototype of hormones, had proved soluble in this way, rather than with the idea of destroying trypsin.

extracts of cat's pancreas were made and prepared for administration in several different ways. Among these, two made in acid media proved more successful when applied to the depancreatized cat than the others and may be presented. Because of the necessity ultimately to prove the combustion of sugar, whatever be the effect on hyperglycemia or glycosuria, we determined to direct our efforts to this objective at once by studying

TABLE I.

*Influence of Extract of Cat's Pancreas on Respiratory Metabolism of Cat. Cat 1.*

Date and time.	Treatment.	Analysis No.	Inspired air.		Expired air.		R.Q.
			CO <sub>2</sub> per cent	O <sub>2</sub> per cent	CO <sub>2</sub> per cent	O <sub>2</sub> per cent	
<i>1931</i>							
Dec. 29		168	0.03	20.92			
		169	0.03	20.93			
" 30							
4.00 p.m.		173			0.26	20.62	0.697
4.15 "		174			0.25	20.63	0.67
	Alcohol check.	175			0.219	20.665	0.666
Dec. 31							
2.30 p.m.		184			0.25	20.625	0.697
3.00 "		184			0.231	20.68	0.717
6.30 "	Given one-fourth of total extract of cat's pancreas. i.v.*						
7.05 "		188			0.299	20.618	0.825
8.00 "		191			0.200	20.72	0.773
8.30 "		192			0.190	20.735	0.763
<i>1932</i>							
Jan. 2	Cat died, no pancreatic tissue.						

\* In this and the following tables i.v. represents intravenous administration; s.c., subcutaneous; i.m., intramuscular; and i.p., intraperitoneal.

effects upon the respiratory quotient first and not to pay any regard to preparations which would not influence the quotient.

The first of these experiments performed on Cat 1 is shown in Table I. The cat had been depancreatized several days before, and both on Dec. 30 and 31 gave good diabetic respiratory quotients. An alcohol check upon the efficiency of the apparatus (Paper I on perfusates (23)) obtained on Dec. 30 likewise is included in the table and shows an unusually perfect

*Effect of Acid Aqueous Extract of Cat's Pancreas on Respiratory Quotient of Cat.*

Cat 2. Pancreatectomy. Feb. 20, 1922.

Date and time.	Analysis No.	Air entering chamber.		Air from chamber.		R.Q.	
		CO <sub>2</sub> per cent	O <sub>2</sub> per cent	CO <sub>2</sub> per cent	O <sub>2</sub> per cent		
1922							
Feb. 20	Alcohol check.	582 592 597	0.03 per cent	20.92	1.20 1.03	19.29 19.54	0.669 0.664
" 22	Depancreatized cat.	603 604 606 607			0.314 0.291 0.263 0.283	20.52 20.55 20.61 20.59	0.660 0.669 0.706 0.722
					Average.....	0.689	
" 23							
1.00 p.m.		611 612		0.273 0.252	20.60 20.63	0.711 0.674	
					Average.....	0.695	
3.35 "	Cat received 15 gm. dextrose by stomach tube.						
4.30 "		614 615		0.273 0.271	20.58 20.58	0.673 0.670	
Feb. 24							
4.00 p.m.	Cat received 10 cc. extract of cat's pancreas subcutaneously after 15 gm. dextrose in stomach.						
5.00 "		617		0.293	20.57	0.721	
5.25 "		618		0.273	20.60	0.712	
5.45 "		619		0.260	20.63	0.774	
6.10 "		620		0.270	20.65	0.896	
6.20 "	Cat received 20 cc. extract filtered through Lloyd's reagent.						
7.15 "		622 623 624		0.292 0.252 0.262	20.60 20.61 20.60	0.794 0.674 0.683	
Feb. 25							
4.00 p.m.	Cat received 30 gm. dextrose by stomach tube.						
5.15 "		632		0.331	20.52	0.711	
7.00 "		635		0.303	20.56	0.714	
7.20 "		636		0.322	20.53	0.711	
8.00 "	Cat received 30 cc. pancreatic extract subcutaneously.						
9.58 "		637 638	0.03	0.344 20.93	20.63	1.12(1)	
10.25 "		640 641		0.463 0.612	20.36 20.16	0.73 0.73	
11.00 "	Cat died.						

agreement with the theoretical quotient. On the 2nd day another cat was depancreatized and the entire pancreas was immediately rubbed up with broken glass in 0.2 N HCl. After thorough maceration and extraction which continued for at least an hour the extract was neutralized with 0.1 N NaOH and was quickly boiled and filtered. The total yield of the clear extract was not recorded, nor the exact amount given the animal, but notation was made that the dose represented one-fourth the entire product. Administration in this case was by means of a cannula inserted under local anesthesia into the femoral vein, and was completed at 6.30 p.m. The cat was immediately placed in the respiration chamber. At 7.05 p.m. the first analysis of chamber air was made and the resulting R. Q. was found to be 0.825; the next, at 7.30 p.m., was 0.77; and the third, at 8.30 p.m., 2 hours after administration, was 0.76.

The second experiment (on Cat 2) is shown in Table II. Pancreatectomy was performed on Feb. 20 and on Feb. 22 the R. Q. showed a condition of nearly total diabetes. To prove this beyond a doubt by the R. Q. a good dose of dextrose was given at 3.35 p.m. on Feb. 23 and the two quotients obtained at 4.30 and 4.55 p.m. were even lower than those obtained before the dextrose was fed. The animal was therefore ready for trial of the extract. The entire gland from another cat was rubbed up with normal saline solution, later acidified to about 0.1 N with HCl and after a couple of hours' extraction was neutralized and, *without boiling*, a portion of the extract was filtered off. Because there was considerable turbidity left the remainder was clarified with Lloyd's reagent before filtering. This seemed to remove a considerable part of the potency also. The entire yield of clear extract was only 30 cc. One-third of this, 10 cc., not clarified with Lloyd's reagent, was given subcutaneously at 4 p.m. on Feb. 24, after 15 gm. of dextrose had been placed in the cat's stomach. In four successive analyses after the cat had been placed in the chamber at 4.20 p.m. the quotient was above the true diabetic level obtained the day before and rose finally to 0.896, 2 hours after the extract was given. At 6.20 p.m. the cat received the remaining 20 cc. of the extract (filtered through Lloyd's reagent). There was only one high quotient after this—0.794 obtained at 7.25 p.m.—the next two showing a return to complete diabetes. The single high quotient was doubtless a hold-over effect of the first injection, and the second injection was ineffectual. Later it will be seen that filtration through charcoal likewise removes the hormone. The very sudden drop to the diabetic level has been witnessed a number of times and will be commented on later.

On Feb. 25 a second extract was prepared in the same manner as the first, but without using Lloyd's reagent, and yielded 40 cc. In a preliminary respiration period subsequent to the ingestion of 30 gm. of dextrose at 4.50 p.m. several successive analyses were in satisfactory agreement as showing the cat's inability to oxidize sugar to any appreciable degree. At 8 p.m. 30 cc., or three-fourths of the entire yield from the single pancreas extracted, were given subcutaneously and the cat was immediately placed in the chamber. The first sample of air terminating at 9.58 p.m. showed the

surprisingly high quotient of 1.12. To confirm the accuracy of the analysis an outside air was run immediately and gave perfectly theoretical figures (see Table II). But the effect was short-lived—in fact almost explosive in its intensity. Moreover, the dose was fatally toxic and the next quotients obtained at 10.25 and 10.45 p.m. were those of partial asphyxia. The cat died at 11.00 p.m.

This second experiment on Cat 2 would not be presented for its evidence of combustion, if it did not also illustrate the toxic effects of these simple extracts when given in large doses. Cat 1 also died (next day) as the result of a similar extract, and it was in the hope of producing a less toxic solution of the antidiabetic substance that we turned to the perfusion method soon after the first experiment recorded above and, with the exception of a very few experiments of which the last described was one, we continued to prefer it as a method of preparation up to June 1, 1922.

It can scarcely be denied that these two experiments taken in conjunction with those performed by Murlin and Kramer in 1916, prove the effect of pancreatic extracts made in dilute hydrochloric acid and neutralized (to litmus) before administration, to raise the respiratory quotient. There was nothing in any of these experiments to indicate that this rise could have been attributable to any other cause than the genuine combustion of sugar. There was no dyspnea or hyperpnea and in none of the experiments just alluded to was any sodium carbonate or bicarbonate employed for neutralizing the acid. Hence there could not have been any "Auspumpung" or washing out of CO<sub>2</sub> through overventilation of the lungs nor any liberation of CO<sub>2</sub> from bicarbonate upon contact with organic acid. Referring, for example, to the very high quotient obtained in the second experiment above, if the extra amount of CO<sub>2</sub> were conceivably due to overventilation (and the animal was under direct observation all the time) it should have been followed in the next period by a compensatory storage of CO<sub>2</sub>, such as is invariably observed in infants after a hard fit of crying, with consequent depression of the R. Q. below the diabetic level.

Nothing concerning the methods<sup>3</sup> employed by Banting and Best, beyond the fact conveyed in personal conversation by Mac-

<sup>3</sup> It is fair to point out that the first experiment reported here was actually performed 3 weeks previous to the first experiments on the respiratory quotient reported by the Toronto workers (Jan. 21, 1922).

leod that the atrophied pancreas had been employed, was known to us until their first paper (19), delayed in reaching our laboratory, was received in April, 1922. On the 27th of May, 1922, Dr. Banting paid a visit to Rochester and from personal conversation it was learned in just what manner acidulated alcohol had been used by them in the preparation of extracts of whole pancreas.

#### *Extracts of Pancreas of Foreign Species.*

Up to this time no attempt had been made by us to prepare extracts or perfusates for use with the human diabetic. But encouraged by the report of Banting and Best and their colleagues, we now began at once to study methods (24) of extraction and purification with a view to the practicability of their use for the relief of the human disease. Obviously, the packing house products would become the major dependence as a source of insulin. We, therefore, began parallel studies of extracts and perfusates (25) of the pancreas of the pig and the steer. Banting and Best seemed to have obtained their best results from alcoholic extracts. We naturally wished to compare this method both for potency and toxicity with the acid aqueous extracts which had already yielded promising results.

Our first efforts to obtain potent but non-toxic extracts from fresh slaughter-house material were not successful because the exact reaction at which the proteins are precipitated upon neutralization seems to be different for the pancreas of different species. After a few weeks, however, this point in the technique was mastered so that water-clear filtrates containing very little protein but containing, if not all, at least a very substantial fraction of the antidiabetic potency could be prepared quite rapidly. They were administered to completely depancreatized dogs in a variety of ways, four of which are illustrated in Tables III and IV and Chart 1. Two dogs (Nos. 1 and 2) were studied simultaneously. The extracts were given unconcentrated and without removal of the salt resulting from neutralization of the acid used in extraction. The member of our laboratory team who had the best mastery of the air analysis having been obliged to withdraw on account of illness, and while another was being trained especially for this work, the respiratory quotient was neglected and attention was concentrated upon the criteria of blood sugar and the D:N ratio.

The first comparisons were made between subcutaneous and intravenous administration, and between acid and alcoholic extracts. Extract 21, used with both these dogs, was prepared from dog's pancreas by maceration with ground glass in 0.2 N HCl. The number in parentheses (1) and (2) in our system refers to the extraction. Thus No. 21 (2) is a second extraction of the same pancreatic tissue as was used in No. 21 (1). Usually a dog's pancreas weighing 25 to 30 gm. was extracted in 250 cc. of the acid. The first dose of 25 cc. of Extract 21, prepared as just described, when given to Dog 1 intravenously, lowered the blood sugar within 3 hours to half its original level and a second dose of 20 cc. given subcutaneously at 12 m. brought down the sugar by 4 p.m. nearly to normal (0.144). The D:N ratio was not established early in the day, but at 11.45 a.m. it was 2.18, and at 3.55 p.m. it was 2.14. There was up to this time, therefore, no perceptible change. In the night urine, however, as determined next morning there was but a small quantity of sugar present, giving in comparison with the nitrogen the low ratio of 0.27. This was one of the earliest observations we had made that hyperglycemia is reduced as a rule well in advance of the descent of glycosuria.

The result showed no perceptible difference between intravenous and subcutaneous administration of the extracts. With Dog 2, however, on the same day (June 23) there was a much slower effect from use of Extract 2B (1) subcutaneously than when it was given intravenously. This extract was prepared from fresh pig's pancreas by extraction in acidulated alcohol after the manner described by Banting and Best (26), and probably because the technique of preparation in this manner was still very new to us it was not very potent—in fact, not so potent as the wholly unconcentrated extract (No. 21) of dog's pancreas, proportionally much smaller in weight, used with Dog 1. Intravenous administration of 25 cc. lowered the blood sugar in 2½ hours from 0.384 to 0.258 and a second dose of 20 cc. given subcutaneously brought it down only a little farther in 4 hours more. There was a more rapid descent of the D:N ratio. Starting at 2.82 at 1 p.m., it fell to 0.96 at 3 p.m. and 0.35 at 4.40 p.m. The night urine, however, showed a complete return to the condition of total diabetes. Whether this difference in the behavior of Dogs 1 and 2 was due to individuality of reaction or to difference in the mode of extraction was not then, and is not yet, entirely clear.

On June 24, 200 cc. of Perfusate 17 (Paper II on perfusates (27)) were given after neutralization by stomach tube to Dog 1 and on the same day Dog 2 received in the same manner 38 cc. of the first extract of dog's pancreas, No. 21 (1). Neither of the preparations given in this manner proved very effective. That given to Dog 2 reduced the blood sugar not more than 60 mg. and in this case there was no material reduction of the D:N ratio. On June 25 no treatment was given to either dog. On June 26 at 11 a.m. both animals were given subcutaneously an injection of acid extract made exactly neutral to litmus. Extract 24 given to Dog 1 was made from two dogs' pancreases. No. 25, given to Dog 2, was made from 1,000 gm. of fresh pig's pancreas trimmed free of fat as nearly as possible. The former had

Dog 1. Pancreatectomy. June 24, 1924.

Date.	Food.	Treatment.			Blood sugar.			Urine.			Remarks.
		Amount. oz.	Time. Extract No.	Method.	Time. L.	Amount. per cent.	Time. gm.	D.N. Nitrogen. gm.			
June 23	100 gm. ground beef. 9.03-9.13 a.m.	25	21 (1)	i.v.	9.00 a.m. 10.05 " 10.35 " 11.50 "	0.405 0.268 0.312 0.206					
	12.00 m.	20	21 (1)	s.c.	1.20 p.m. 2.00 "	0.207 0.180	11.45 a.m. 3.22	1.482.18			
					3.55 p.m.	3.57	1.622.14				
June 24	200 gm. ground beef. 9.00 a.m.	200	17 Perfusate neutral- ized.	Stomach tube.	9.00 a.m. 10.20 " 11.20 " 1.50 p.m. 3.50 "	0.299 0.282 0.322 0.275 0.279	Night. 3.00 p.m.	5.62 1.443.90	1.380.27		
				No treatment.							
June 25	300 gm. ground beef.	25	24	s.c.							
June 26	300 gm. ground beef.	11.00 a.m.	Neutral- ized.				10.55 a.m. 12.00 m. 1.10 p.m.	0.251 0.285 0.285	6.45 2.182.91		



TABLE IV.  
Dog 2. Pancreatectomy. June 21, 1922.



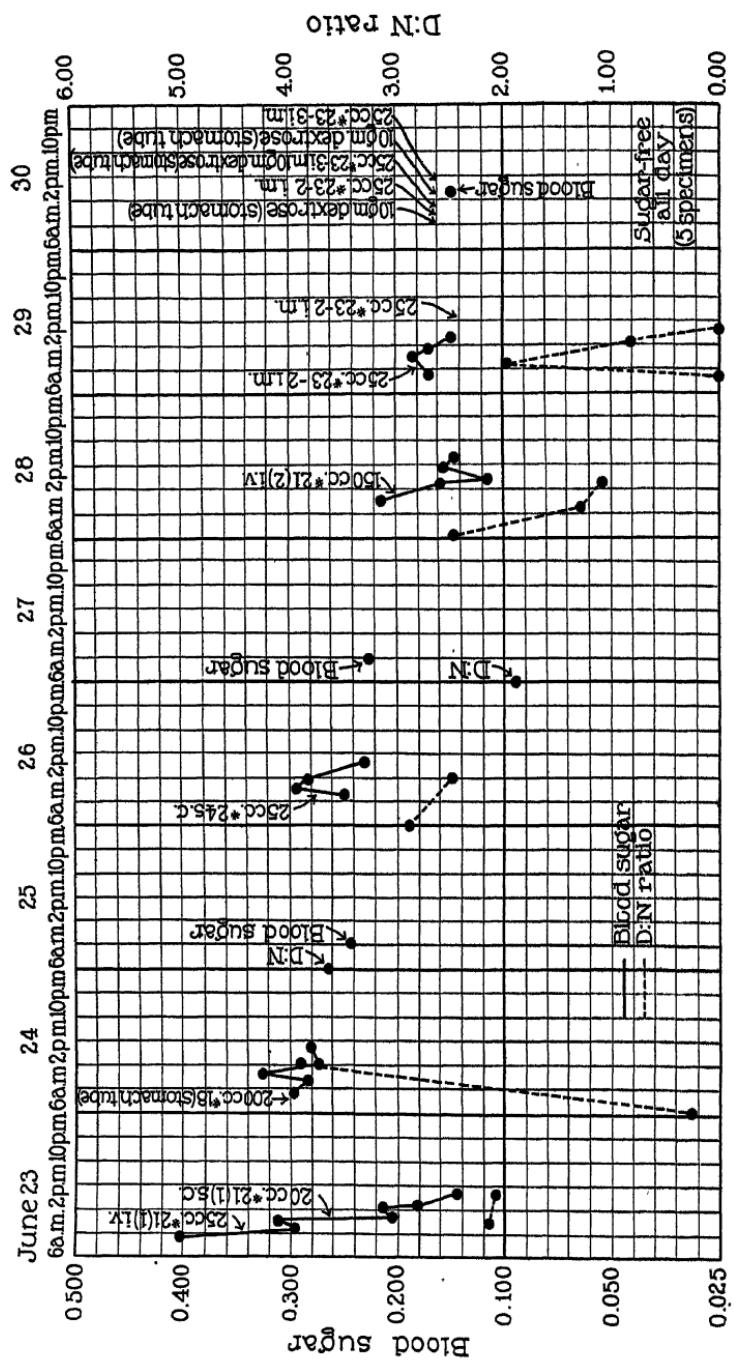


CHART 1. Comparison of different methods of administration of pancreatic extract. Dog 1. In this and the following charts .v. represents intravenous administration; s.c., subcutaneous; i.m., intramuscular; and i.p., intraperitoneal. The number of the extract and quantity given are shown in the chart.

extracted for 3 days, the latter only 2 days. In neither case was the effect very pronounced, probably because the neutralization had not been carried to exactly the right point. No treatment was given on June 27.

*Effect of Boiling.*—On June 28 Dog 1 received intravenously 150 cc. of extract of dog's pancreas, No. 21 (1), brought rapidly to the boiling point, neutralized to a very faint acid reaction to litmus, and filtered. The infusion was conducted very slowly, requiring 55 minutes. In 1½ hours from completion of the injection the blood sugar had fallen from 0.217 nearly to normal (0.119) and it remained low at least until 4.30 p.m. The D:N ratio had already fallen at 2.50 p.m. and the night urine collected next morning was entirely free of sugar. This experiment confirms the original observation of Murlin and Kramer in 1913 where the effect was erroneously ascribed to the influence of alkali; for with two unimportant exceptions the procedure was the same. In the earlier experiment extract of duodenal mucosa had been included while in the present case only pancreas was used. While at the time it was thought the duodenal mucosa might have contributed to the result, it is now certain that the pancreatic extract alone was sufficient to account for the disappearance of sugar from the urine. In the former experiment also sodium carbonate had been used for neutralizing the free acid remaining after extraction; while in the experiment now before us the alkali employed was sodium hydroxide. There is no reason to believe this difference is material; for in neither case was more than enough alkali used to neutralize the acid.

It is clear from these results and from several other similar experiments (see for example Dog 3. below) that insulin is not destroyed by rapid boiling in acid medium.

The next extract employed with Dogs 1 and 2 (No. 23 (2)) was prepared as nearly as possible after the manner described by Banting and Best with acidulated alcohol. Pig's pancreas freed as much as possible from fat and weighing 1,952 gm. was placed in 4,000 cc. of 95 per cent alcohol plus 16 cc. of concentrated HCl, sp. gr. 1.19, making approximately 0.18 per cent HCl. Extraction continued for 6 days when 450 cc. of the clear alcoholic fluid were evaporated to dryness, taken up in 225 cc. of sterile Ringer's solution, and shaken with ether for removal of fat. The extract was allowed to stand in the separatory funnel over night when it was separated from the ether layer and was placed in sterile 1 ounce bottles for storage in the ice box.

Several injections of this preparation were made intramuscularly into both dogs. There was a rapid fall in blood sugar in Dog 1 (not determined in Dog 2) and the urine became sugar-free, remaining so in Dog 1 for 2 days. Insulin, therefore, undoubtedly was present. But the preparation was fearfully toxic and both dogs died as the result. Autopsy revealed areas of gas and fluid under the skin at the sites of injection and extensive gelatinization or liquefaction or both of the muscles. The condition suggested enzyme action possibly due to incomplete destruction of trypsin.

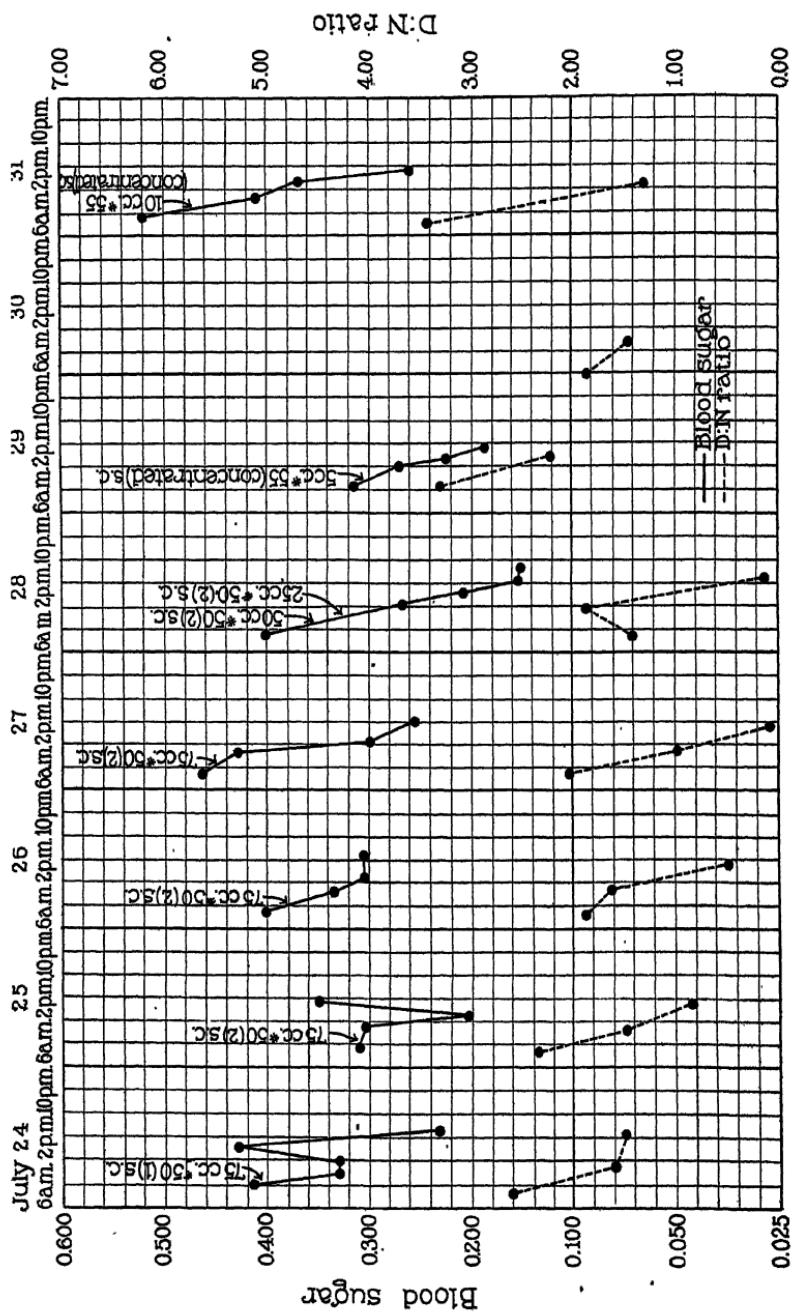


CHART 2. Comparison of first and second extractions from the same pancreas, unconcentrated, given subcutaneously to Dog 12; also, concentrated extract.

*Autopsy*.—Dog 1. Extreme emaciation. Groins icteric. Operative wound in excellent condition, practically healed. Few drops of free pus on incision. *Abdomen*.—Large amount of free fluid (200 cc.?), reddish brown in color. Liver adhesions to body wall. Entire liver coffee-colored. Site of pancreatectomy normal. Mesentery shows blackish injection (postmortem?). *Thorax*.—Small amount of free fluid like that seen in abdomen. Viscera normal. *Sites of Injection*.—(a) *Right Hip*.—Gas crepitation. Considerable yellowish brown fluid. (June 29, 25 cc. of No. 23 (2) intramuscularly.) (b) *Right Shoulder*.—Tissue necrotic. Pocket of fluid over humerus. (June 30, 25 cc. of No. 23 (2) intramuscularly.) (c) *Left Shoulder*.—Necrosis with yellow fluid (necrotic fat?). (June 30, 25 cc. of No. 23 (2) intramuscularly.) (d) *Left Lumbar*.—Necrosis with fluid. (July 4, 25 cc. No. 25 (1) subcutaneously.)

*Autopsy*.—Dog 2. Marked edema of entire left fore leg, extending to foot and over entire left thorax. Crepitation in axilla. Area of gas and fluid in right groin. Operative incision appears to be in fair condition, gaping slightly. Lower layers of subcutaneous tissue of thorax appeared gelatinous when incision was made and some light brown fluid escaped. Sanguinous fluid escaped from lower portion of abdominal incision. Wound infected down to peritoneum, which was smooth and glistening with no evidence of infection. *Abdomen*.—Periphery of entire mesentery appeared black, resembling India ink staining. This discoloration was not seen in the main branches of the mesenteric artery. Discoloration most marked in region of cecum where numerous old adhesions were found (probably from oophorectomy?). Site of operation in good condition. Two ligatures were found, one near the former location of the head of the pancreas and the other near the tail. The mesentery had established firm union with the intestine along the line of dissection of the pancreas. There was no evidence of infection. The left kidney was more congested than the right—apparently an hypostatic change. Gall bladder and liver normal. Stomach normal and empty. Small intestine considerably distended with gas but otherwise normal. Urinary bladder empty. *Thorax*.—Both pleural cavities contained dark brown colored fluid with peculiar (not putrefactive) odor. In this fluid there were many small black flecks. At least 100 cc. of this fluid in each pleural sac. The tip of the lowest lobe of the left lung was black for about 1 inch. The portion of diaphragm under this area was everywhere glistening and normal in appearance. Heart normal. *Sites of Injection*.—(a) *Left Axilla*.—Extensive necrosis of all muscle in this region. Muscle dark red and reduced to a pulp which was easily scraped away. The muscle tissue appeared to have undergone extensive digestion. No purulent fluid present, but considerable foul smelling gas escaped when incision was first made. Necrotic tissue extended through the latissimus dorsi and down into the left thoracic wall. Reddish brown fluid escaped freely. The condition suggested autolysis rather than putrefaction. (June 29, 25 cc. of No. 23 (2) intramuscularly.) (b) *Thighs*.—Incision into the muscle of the thighs showed beginning necrosis. These muscles were more gelatinous and did not show the deep

purplish red that was seen in the muscle of the axillary region. (June 30, 25 cc. of No. 23 (2) intramuscularly.) *Impression.*—Extensive autolysis of muscle tissue at the sites of the intramuscular injections of No. 23 (2).

*Other Toxic Effects.*—Following the experience just related with Dogs 1 and 2 a number of other dogs were lost as the result of toxic effects produced by extracts administered while still acid in reaction. As illustrations of these effects the autopsy findings in the cases of Dogs 4 and 5 are given below.

Dog 4, a small white female, was operated on June 29, 1922. On July 1 and 2 several intramuscular injections were given of Perfusate 19 made from pig's pancreas, but given without neutralization of all free acid. The dog became so edematous that it was chloroformed on July 5 and immediately examined.

*Autopsy.*—Dog 4. Gas over all areas of injection. Marked sloughing at both operative incisions. *Abdomen.*—Free fluid in abdomen (purulent, thin) with beginning peritonitis. Plastic adhesions around site of pancreatectomy with necrosis of tissue of glandular nature (pancreas?). Suture in midst of tissue. Mesentery injected. Operative field otherwise in normal condition. *Thorax.*—No free fluid; negative. *Sites of Injection.*—(a) *Right Shoulder.*—Tympanitic to percussion. Agar tube inoculated and plate poured. Muscles gelatinous; fat appears liquefied. Gas between muscle layers extending into axilla. Crepitus all way down humerus. (July 4, 25 cc. of No. 25 (1).) (b) *Right Hip.*—Gas in all muscle layers. Reddish brown fluid present. Muscle tissue necrotic. Fleshy odor to fluid. (July 1, 25 cc. of No. 26.) (c) *Left Hip.*—Profuse chocolate-brown fluid. Odor not purulent. (July 1, 25 cc. of No. 29.)

Dog 5 was depancreatized July 7, 1922. On July 9 two injections were given of Extract 35 made from pig's pancreas in 0.2 N acetic acid. The extract was given after neutralizing all the free acetic acid except 0.1 per cent. The dog was found dead next morning.

*Autopsy.*—Dog 5. Sanguinous fluid escaping from abdominal wound. Crepitation in both groins and over outer part of both thighs. Abdominal wound broken down. *Abdomen.*—Mesentery necrotic (perhaps postmortem change) and beginning to adhere along lines of pancreatectomy. No free fluid; no sign of peritonitis. Some angulation of intestine near pylorus, but no change in appearance of wall. Probably no obstruction. Pancreatectomy wound in good condition and apparently healing. *Thorax.*—Considerable postmortem change. Lungs crepitant. No pericarditis or pleuritis. *Groin.*—Surface of muscles of right groin reddened and gelatinous—more than left. *Right Thigh.*—Huge descending pocket extending towards tail and towards groin. Filled with necrotic and sloughing tissue and gas. Evidence of hemorrhage into this pocket. (July 9, 25 cc. of No. 35 (1).) *Left Thigh.*—Pocket like that on right side, muscle gelatinous and so necrotic that it could be scraped away with the scalpel in the form of a thick paste. (July 9, 25 cc. of No. 35 (1).)

*Control of Reaction All Important.*—The extremely serious consequences of failure to destroy the trypsin completely, illustrated by the fatal results with Dogs 1 and 2, or to neutralize practically all the free hydrochloric acid, illustrated by the death and autopsy reports upon Dogs 4 and 5 cannot be overemphasized. It is quite immoral scientifically to give out the impression that no danger is to be feared from imperfectly prepared extracts and the public as well as the profession should receive warning of the misrepresentations of quacks who almost certainly will seek to exploit this new remedial agent and foist upon the public improperly prepared remedies.

Gas formation (which proved on direct examination to be  $\text{CO}_2$ ) was the most common result of free HCl. Edema, muscular stiffness, and necrosis also were encountered. Control of the reaction to about 0.01 per cent HCl, however, obviated these pathological effects.

*Intraperitoneal Administration.*—The end of our difficulties on account of the toxic effects of free HCl came about through attempts to administer extracts intraperitoneally. Given subcutaneously, intravenously, or even intramuscularly there was no manifestation of pain even when the acidity was 0.05 per cent; but when intraperitoneal injection was undertaken the pain was intense. Careful adjustment of the reaction to very near the neutral point to litmus (pH 3.5 to 6.5) annulled the pain and still gave very positive antidiabetic results. Dog 3 (Table V) illustrated these progressive steps. The various injections given to this dog are numbered in the table. The first two made from dogs' pancreases were given after neutralization to 0.05 per cent HCl. As noted in the column of Remarks much crepitus and gas formation at the sites of injection resulted. Drawing some of this gas into a test-tube fitted with clear baryta solution caused a very heavy white precipitate, proving its identity as  $\text{CO}_2$ . When the dog had developed a sufficiently advanced stage of diabetes as shown by the blood sugar level of 0.289, several injections of boiled extract of pig's pancreas were given, two of them subcutaneously and one intraperitoneally. All were potent though not so potent as unboiled extracts.

Extract 39 made from beef pancreas in 0.2 N HCl and neutralized exactly to 0.05 per cent HCl given intraperitoneally on July 14 caused a rapid decline of the hyperglycemia and complete sup-

TABLE V.  
*Effects of Extracts of Dog, Pig, and Ox Pancreas.*  
 Dog 3. Pancreatectomy. July 10, 1922.

Date.	Food.	Treatment.			Blood sugar.			Urine.			Remarks.
		Determination.	Time.	Amount.	Method.	Time.	Amount.	D:N.	Nitrogen.		
1922											
July 10	None.	(1)	5.30 p.m.	20 cc.	37 S.C.						
July 11	None.	(2)	11.55 a.m.	25	31 Neutralized to 0.05 per cent HCl.	S.C.					
July 12	100 gm. beef heart.	(3)	10.05 a.m.	30	43-B Boiled.	S.O.	9.45 a.m. 11.20 "	0.289 0.205	2.800.84	Much crepitus over sites (1) and (2). CO <sub>2</sub> obtained from pockets.	
		(4)	12.30 p.m.	25	43-B Boiled.	S.O.	12.32 p.m. 1.20 p.m. 5.00 "	0.207 0.242 0.268	1.00 p.m. 0.81 0.591.37		

July 13	6.25	200 gm. beef heart.	(5)	10.25- 10.40 a.m.	150	41-B Boiled.	i.p.	10.15 a.m.	0.281	6.30 a.m.	2.53	2.31	1.10	No toxic effects from (3) and (4) injections. Necrosis from (1).	
								11.45 "	0.274	8.05 "	1.34	0.41	3.24		
								12.45 p.m.	0.194						
								1.45 "	0.266	3.30 p.m.	3.13	2.7	1.16	Severe pain.	
								4.00 "	0.255						
July 14		Beef heart. Ate very little.	(6)	11.00 a.m.	200	39 (2) Neutralized to 0.05 per cent HCl.	i.p.	10.50 a.m.	0.237	6.30 a.m.	2.01	1.2	1.7	Hemoglobin per cent	
								12.00 m.	0.188	11.00 a.m.	4.8	2.3	2.1	10.50 a.m.	
								1.00 p.m.	0.157					55	
								3.40 "	0.116					66	
								6.00 "	0.157						
July 15	5.79	150 gm. beef heart. Ate all.	(7)	11.20- 11.34 a.m.	147	39 (2) Neutralized to 0.05 per cent HCl. 10 mg. novocaine.	i.p.	11.15 a.m.	0.240	6.30 a.m.	0	10.30 "	1.17	1.07	1.1
								12.30 p.m.	0.238						
								1.30 "	0.192						
								3.30 "	0.108	4.05 p.m.	0				
July 16	5.62	160 gm. beef heart.				No treatment.				6.30 a.m.	0				
										6.30 p.m.	0				

TABLE V—Concluded.

Date.	Food.	Treatment.			Urine.			Remarks.
		Determination.	Weight.	Time.	Blood sugar.	Time.	Nitrogen.	
1922								
July 17	5.67 gm. beef heart. (8)	Amount: 90 Bacteric. No.: 38 (2) Method: Exactly neutralized to litmus, filtered.	oz.	i.p. 9.45 a.m. 11.00 " 0.154 12.00 m. 0.136 2.00 p.m. 0.107 4.10 " 0.123	per cent 0.235 " 0.154 " 11.00 " 1.25	6.30 a.m. " 1.20 " 0.595 " 1.04	0.6 0.04 1.04	No pain.
July 18	5.67 gm. beef heart. (9)	Amount: 75 Bacteric. No.: 38 (2) Method: Exactly neutralized to litmus. Filtered through charcoal.	oz.	i.p. 11.30 a.m. 2.30 p.m. 4.30 "	0.260 0.240 0.260	6.30 a.m. " 1.20 " 0.46 " 0.85	0.6 0.6 0.6	Fluid obtained from cavity cultured.
July 19	5.45 gm. beef heart. (10)	Amount: 65 Bacteric. No.: 38 (2) Method: Exactly neutralized. Filtered through paper.	oz.	i.p. 11.00 a.m. " 1.20 " 0.258 " 0.242 " 0.200	1.62 1.6 1.6 No pain.	10.30 a.m. " 1.20 " 0.46 " 0.90	1.4 1.6 1.6	Culture of July 18 apparently <i>Staphylococcus albus.</i> No pain.

July 20	5.35	40 gm. beef heart.	(11)	10.35 a.m.	50	48 (1) + 5 per cent alcohol.	s.c.			6.30 a.m.	1.56	0.89	1.8
				4.35 p.m.	45	Same.				9.15 a.m.	Trace only of sugar.		No reaction from injection (11).
July 21		Did not eat.											

Aug. 4 Dog died of inanition and terminal pneumonia.  
*Autopsy.*—Abdominal wound slightly gaping. Purulent discharge. Pancreatectomy wound perfectly repaired, mesentery firmly attached. Intrabdominal fat has entirely disappeared from mesentery and around kidneys. Peritoneum in normal condition. All abdominal viscera appear normal. *Thorax.*—Lungs, right upper and middle lobes show pneumonic condition, seropurulent discharge from lung on section. Left lung appears normal. Emaciation marked. *Sites of Injection.*—Large burrowing pockets over both hips (injections (1) and (2)). Sites (injection (11)) along spine all normal.

pression of the glycosuria. Because a large amount of fluid was used in this injection it was uncertain whether a part of the drop in percentage of blood sugar might not be due to dilution of the blood. Hemoglobin determinations were made by means of the Dare apparatus and proved conclusively enough that the dilution was compensated by elimination of water through the kidney. Moreover, the same effects were obtained on July 15 from a smaller injection of the same preparation.

The first of these injections was extremely painful; the second somewhat less so on account of the novocaine introduced with the first few cubic centimeters of the extract. Extract 38 (Injection 8) made from fresh beef pancreas in 0.5 N HCl, but neutralized to within less than 0.01 per cent HCl proved almost painless and yet very potent. Thereafter all our unconcentrated extracts were neutralized to within the range mentioned, and there have been no further toxic results such as those described in the autopsy reports for Dogs 4 and 5. Induration occasionally has been encountered, but no fatal toxemias. The abscesses which developed following acid injections were frequently cultured but only occasionally, as in Dog 3 on July 18, have they yielded any growth on agar.

This table illustrates one further characteristic of insulin. Preparation 38 (2) was filtered through charcoal before administration on July 18 and proved entirely impotent to affect the blood sugar or to diminish the excretion of sugar. This has been confirmed repeatedly. Charcoal, therefore, should prove to be a satisfactory absorbent for insulin.

#### *Comparison of Alcoholic and Aqueous Acid Extracts.*

One animal may be selected to illustrate the relative potency of extracts made on the one hand by extraction with acidulated (0.2 per cent) alcohol after the manner described by Banting and Best, and on the other by extraction in aqueous medium made acid to 0.2 N by addition of HCl, after the manner first used by Murlin and Kramer. Table VI containing one such comparison with Dog 6 is given. Pancreatectomy was performed on July 21 and on the 3rd day the blood sugar and the D:N ration indicated a condition of severe diabetes.

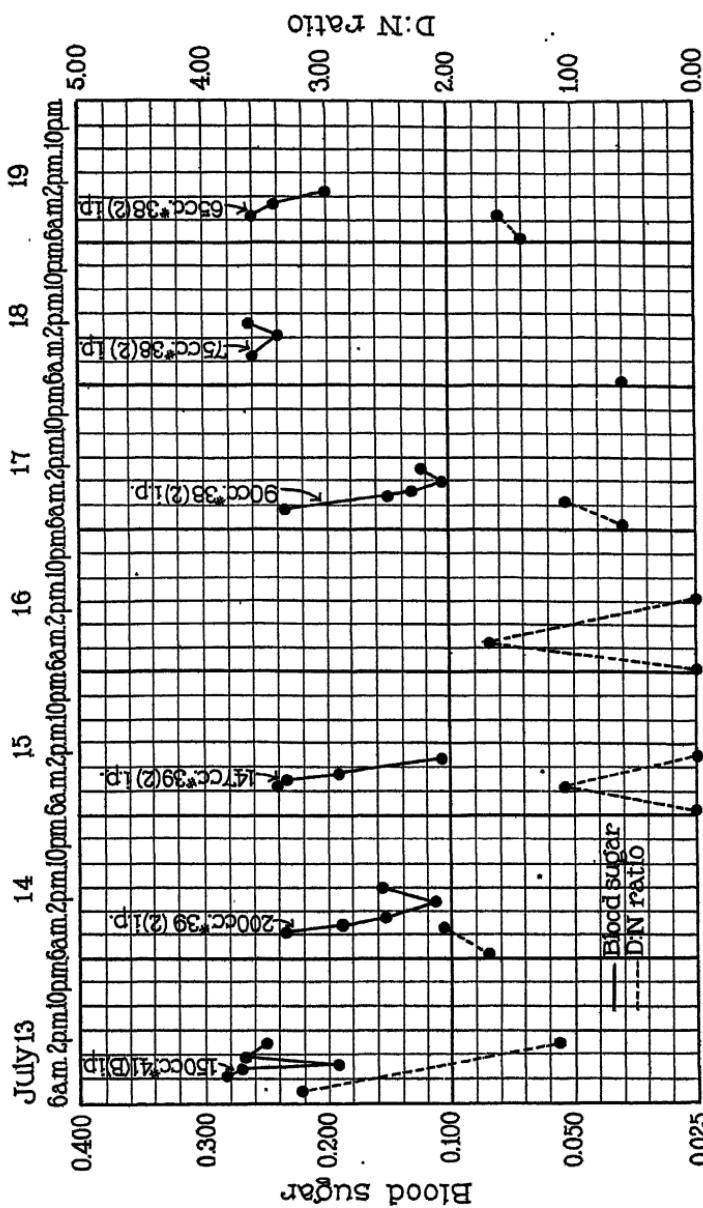


TABLE VI.  
Dog 6. Date of operation July 21, 1922.

Date 1922	Weight, kg.	Diet.	Treatment.			Blood sugar.			Urine.			Remarks.
			Amount, cc.	Title,	Method.	Extract No.	Title,	Amount, per cent	Sugar, gm.	Nitrogen, gm.	D.N.	
July 21												
July 22	12.7		4.00 p.m.	Pancreatectomy.					6.30 a.m.	Medium amount.		No complications.
July 23	12.4	80 gm. ham- burg.						9.30 "	"	Medium amount.		Vomited during night. Condition good.
July 24	12.0	150 gm. ham- burg.		Catheterized.				6.30 a.m.	Heavy.			Condition good. Cath- eterized.
			10.08 a.m.				10.15 a.m.	0.207	9.40 "	3.60 2.94	1.22	
			10.18 a.m.	75 (50/2) 3 places.	s.c.		12.15 p.m.	0.279		10.08 a.m.	0.8510.3182.68	Condition good.
							2.15 "	0.215			gm. per hr.	Wound dry, stay stitches cutting.
			2.08 p.m.	Catheterized.			4.20 "	0.184				
							6.35 "	0.105				
									6.40 p.m.	Sugar-free.		
July 25	11.8	Does not eat.		Catheterized.								Condition and wound good.
			9.50 a.m.				9.55 a.m.	0.258	9.50 a.m.	0.7890.4801.64		
			11.15 a.m.	25 (46 (1)	i.v.		1.20 p.m.	0.167	1.50 p.m.	0.4800.2941.67		
							3.20 "	0.108				
							5.20 "	0.087	5.50 p.m.	Sugar-free.		

Extract 50 (2) used on this dog was prepared as follows: On July 19 a refrigerated vessel, containing distilled water chilled to below 0°C., was taken directly to the slaughter-house. Pancreases of five animals as rapidly as they were taken from the carcasses, were trimmed free of extraneous tissue and immediately dropped into freezing water. The freezing mixture was carried by automobile at once to the laboratory where the organs were passed through a meat grinder. The entire mass, weighing 1,400 gm., was then placed in 5,600 cc. of 0.2 N HCl and the vessel placed in an ice box directly in contact with ice. 2 days later this liquid was poured off and 4,000 cc. of fresh 0.2 N HCl were added. 3 days later 130 cc. of the supernatant fluid were neutralized with 21.64 cc. of 0.1 N NaOH and allowed to stand a short time for complete precipitation. Upon filtration the fluid portion came through perfectly clear and showed upon test a very slight milkiness with alcohol. Filtration was accomplished aseptically, directly into sterile bottles. There were approximately 150 cc. representing 30 gm. of fresh pancreas.

Preparation 46 (1) used on July 25 was prepared in the following manner: On July 13, 1,770 gm. of pig pancreas, trimmed, nearly free of fat, were placed in 7,000 cc. of 95 per cent alcohol, containing 0.2 per cent HCl. Extraction continued at room temperature until July 21 when 900 cc. of the supernatant fluid were evaporated over a water bath with precautions to keep the temperature well below 30°C. The fat which separated out spontaneously was skimmed off, and the aqueous fluid transferred to a clean evaporating dish and warming continued before an electric fan until the odor of alcohol had disappeared. The volume was now 75 cc. An equal quantity of Ringer's solution was added, the mixture filtered to remove still more fat, and the clear liquid washed in a separatory funnel with ether. The aqueous liquid was drawn off, the excess ether evaporated over the water bath below 30°C., and the final clear product placed in a sterile bottle. The 900 cc. of original extract had been concentrated to 150 cc. and represented approximately 240 gm. of fresh pancreas.

The 75 cc. dose of Preparation 50 (2) given on July 24 represented, therefore, about 15 gm. of fresh beef pancreas, while the 25 cc. dose of No. 46 (1) represented about 40 gm. of fresh hog pancreas. It might be expected, therefore, that the latter would produce a somewhat greater effect, especially as the aqueous preparation represented the second extraction from the same mass of tissue. When this is taken into account it is evident that the acid aqueous method gave results quite as good as the alcoholic method. In both instances the blood sugar dropped to normal, but the drop was more rapid with the alcoholic extract, probably because it was given intravenously, while the aqueous extract had been given subcutaneously. The urine was rendered sugar-free in 8 and 6 hours, respectively.

One other example of the complete competence of the aqueous method of extraction as judged by the effects on blood sugar and urinary sugar may be given. Dog 7 was operated on Aug. 8 and developed a total diabetes on the 3rd day following. For several days thereafter he was used for testing various preparations which proved to be only mildly potent. On Aug. 15 Preparation 62 (1) was given in two doses of 50 cc. each. The mode of preparation was essentially like that of No. 50 (2).<sup>4</sup> The first extraction of which the doses used on this day were part continued for 48 hours. A 100 cc. portion was neutralized to very faintly acid reaction, filtered, and

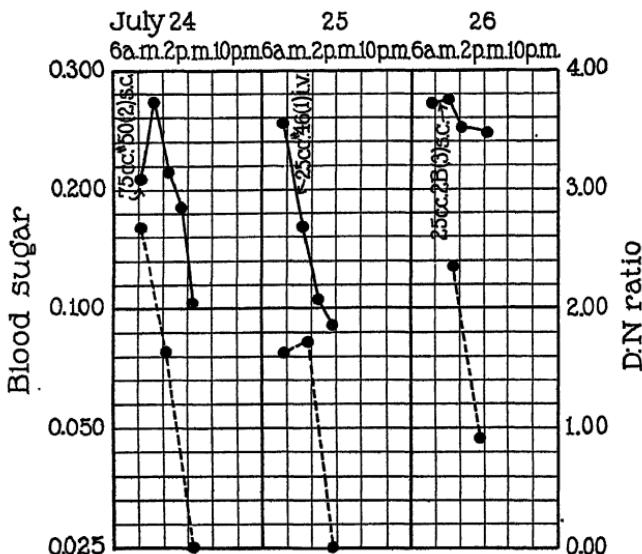


CHART 4. Comparison of acid aqueous and acid alcoholic extractions given to Dog 6.

administered subcutaneously without further manipulation. The effect is shown in Table VII. The blood sugar fell from 0.292 to 0.092 in 8 hours from the time of the first injection. From a D:N ratio of 1.97 in the early morning the glycosuria diminished until at 4.15 p.m. it had entirely disappeared.

The next day two doses of Preparation 63 (2) were given at an interval of 2 hours. This preparation was made from 1,000 gm. of fresh beef pancreas after the manner already described for No. 50. The first extraction was continued for 24 hours and the second extraction of which these doses were part, for 6 days. The effect was even greater than with No. 62 (1)

<sup>4</sup> See page 279.

and illustrated a fact which we have repeatedly confirmed; namely, that the second extraction made in this way contains more insulin than the first. The blood sugar declined from 0.310 to 0.041 in 6 hours from the time of the first injection. From a D:N ratio in the early morning of 0.56 the glycosuria had completely disappeared within the same time and remained absent over night. On Aug. 17 the dose was reduced to 25 cc. and at 2 p.m. 20 gm. of dextrose were given for the purpose of a respiration experiment which because of a temporary defect in the apparatus was not performed. The sugar had overwhelmed the effect of this one dose of extract and as a consequence the blood sugar rose. Sugar also appeared in the urine, but up to 9.30 a.m. the next morning only 8.52 gm. of the 20 gm. fed had been excreted and at 9.50 a.m. a more effective dose of extract began to check the excretion so that only a negligible amount of sugar escaped. It is fair to state that the 25 cc. of extract given at 10 a.m. on Aug. 17 had disposed of (not necessarily caused the combustion of) some 12 gm. of glucose. The single dose of 50 cc. given at 9.50 a.m. on Aug. 18 was sufficient to hold the urine sugar-free throughout the day. It is worthy of remark that Dog 7 lived 41 days after pancreatectomy and finally died by accident.

The only special pertinence of the foregoing experiment as against any one of several others of like purport which might be reported is that these two preparations, Nos. 62 and 63, were later cleared of protein, concentrated, and administered to a human case (26) of diabetes with complete control for the time of the glycosuria and hyperglycemia.

#### *Respiration Experiments.*

The remainder of this paper will be devoted to the presentation of additional evidence that extracts prepared in acid aqueous media can bring about the oxidation of glucose. The only difference between the experiments already cited in proof of this proposition and those to follow is, that in the earlier group the extract employed was made from pancreas of the identical species, while in the latter, made entirely from pancreas of beef, it was used exclusively on the diabetic dog.

It is not necessary here to enter into a discussion of the respiratory quotient of diabetes. That ground has been covered sufficiently in previous papers (23). The quotient with which one prefers to start is that which is typical of total diabetes; namely, 0.68 or 0.69, as illustrated in Tables I and II. However, it is not absolutely necessary to secure this quotient in order to demonstrate a considerable rise following the administration of insulin.

TABLE VII.  
Dog 7. Date of operation Aug. 8, 1922.

Date.	Weight. kg.	Diet.	Treatment.		Blood sugar.		Urine.		Remarks.	
			Time.	Amount. cc.	Method. Extract No.	Time. per cent.	Time. per hr.	Nitrogen. gm.		
1932	10.6	ground beef.	10.00 a.m.	50	62 (1)	8.45 a.m. 8.00 m.	0.292	8.30 a.m. 1.153 0.584	1.97	Condition good. Urine sugar-free, ulcer behind left fore leg. Wound sup- purative.
Aug. 15	10.0	225 gm. ground beef during night of Aug. 16.	12.30 p.m.	50	62 (1)	12.00 m. 2.00 p.m. 4.00 "	0.225 0.176 " 0.125	0.150 0.189 gm. per hr. (much diluted.)	0.79	
						6.05 p.m.	0.092			
Aug. 16	10.0	225 gm. ground beef	8.45 a.m.	Catheterized.		8.45 a.m.	8.55	15.12	0.56	Condition good. Pocket and ulcer behind left leg. Wound purulent.
			11.30 a.m.	50	63 (2)	9.00 a.m.	0.310			
			1.30 p.m.	50	63 (2)	8.00				
						1.41 p.m. 3.35 "	0.141 0.063	1.40 p.m. 5.30 "	13.2	0.42
						5.35 "	0.041	5.30 "		Sugar-free; no reduction with 40 drops.

	Aug. 17	9.76	260 gm. beef (ground)	9.30 a.m. 10.00 "	Catheterized. 25   63 (2) s.c.	9.35 a.m. 0.219 12.05 p.m. 0.215	9.30 a.m. 0.219 2.00 p.m.	Sugar-free with 40 drops.	Condition good. Pocket unchanged.
								No new le- sions at any injection site.	
	Aug. 18	9.1	200 gm. ground. beef at 6 p.m.	9.30 a.m. 9.50 "	Catheterized. 50   63 (2)	9.40 a.m. 0.299 12.00 m. 0.245 2.00 p.m. 0.187 4.00 " 0.128 5.30 "	9.30 a.m. 0.299 1.30 p.m.	9.30 a.m. 0.299 1.30 p.m.	Condition good. Wound healing. No new lesions.
								Sugar-free, no reduction with 40 drops.	
						5.30 p.m.		Sugar-free; no reduction.	

TABLE VIII.  
Dog 8. Date of operation Aug. 28, 1922.

Date.	Weight. kg.	Diet.	Treatment.	Blood sugar.			Respiratory metabolism.			Remarks.
				H. H. H. H.	No. Extract No.	Method. Amount. cc.	Time. per cent	5 H. H. H.	5 H. H. H.	
1922	11.0		7.00 a.m. 25 gm. glucose in 250 cc. water in cage. 10.30 a.m. 100   62 (2)   s.c. in 4 places. 11.15 a.m. 20 gm. glucose in 300 cc. water in bucket.	10.20 a.m. 0.320			9.30 a.m. 4.45 p.m.			(0.72 0.71 0.72) { Condition good. Drank 200 cc. first portion glu- cose.
Sept.	11.0	175 gm. ham- burg during night.								(0.73 0.74 0.72 0.74)
Sept.	11.0	130 gm. ham- burg eaten at 5 p.m.	8.20 a.m. 110   63 (2)   s.c. 10.27- 10.35 a.m. 10 gm. glucose i.y. in 150 cc. water.	10.40 a.m. 0.370			8.00 a.m. 11.45 a.m. 12.05 p.m. 4.40 p.m.	70.29 92.77 70.29 92.77 81.9 102.8 82.7 104.7 71.6 110.7 72.5 110.7	{ { { { { {	Condition fair. Dog seemed slightly worse after i.v. sugar. Breathing slowly.

								Condition improved.
								Condition good.
Sept. 3	150 gm. ham- burg.		No treatment.					
Sept. 4	200 gm. ham- burg.		No treatment.					
Sept. 5								
	10.45 a.m.	30	64 (1)-C s.c.	10.30 a.m.	0.308	9.20 a.m.	{ 97.8 97.8	0.75 0.74
			12.20 p.m. 10 gm. dextrose.	i.v.			{ 141.2 140.0	0.87 0.87
							{ 110.3 110.3	0.81 0.80
							{ 137.2 137.2	0.80

For example, Dog 8 (Table VIII) deprived of the pancreas on Aug. 8, was given 4 days in which to develop complete intolerance for carbohydrate. A test feeding of 20 gm. of glucose given at 7 a.m., Sept. 1, did not raise the R. Q. beyond 0.72. There was, therefore, little or no residual capacity to oxidize sugar. At 10.30 a.m., 100 cc. of Preparation 62 (2), already described, were given subcutaneously in four places, and at 11.15 a.m. another feeding of 20 gm. of glucose in water. The R. Q. at 4.45 p.m. had risen only a couple of points. Other evidence of benefit from the extract, however, was found in a blood sugar level of 0.286 at 4.50 p.m., notwithstanding the 45 gm. of glucose which had been ingested during the day. The next morning at 8 a.m. the quotient was found to be higher (0.76), indicating that the insulin had begun to affect the oxidation of sugar after the test which was made at 4.45 p.m. the day before and that the high point in the curve had probably been passed in the night.

A second injection of No. 63 (2), known from previous evidence to be potent, given at 8.20 a.m. on Sept. 2, and followed at 10.27 a.m. by an infusion of 10 gm. of glucose into the vein, brought up the R. Q. at 11.45 a.m. to 0.80. But the effect was fleeting; for at 1.50 p.m. the quotient had fallen again to the diabetic level. Attention was called to the same phenomenon in the case of Cat 2<sup>6</sup> and had been noted with perfusates (27). One might be inclined to infer a quantitative relationship rather than a catalytic relationship of insulin to the reaction of oxidation.

No further treatment was given Dog 8 until Sept. 5. The night urine of Sept. 4 contained a D:N ratio of 1.21 (not shown in Table VIII) and the R. Q. was found at 9.20 a.m. to be 0.75. Both tests show that the animal at this time was not completely diabetic. No explanation can be offered for this condition unless it was due to a delayed effect of the extract given on Sept. 2. The blood sugar at 10.30 a.m., however, shows plainly enough that whatever protection the animal had received was nearly exhausted. A single injection of Preparation 64 (1)-C (made by extraction in alcohol containing 0.2 N HCl) given at 10.45 a.m. followed at 12.20 p.m. by intravenous infusion of 10 gm. of glucose, raised the quotient to 0.87 at 2.35 p.m., 4 hours after administration of the extract. At 5 p.m. it had fallen again to 0.80.

The observations on this animal illustrate the importance of other contributing factors than the insulin itself in securing evidence of combustion. One of these unquestionably is what may be called the degree of saturation of the tissues (particularly the liver) with glycogen. It is known that with a normal animal which has been fasting for some time a single dose of sugar, which, in a well fed animal would produce a R. Q. approaching unity, may fail to show any effect and the interpretation is that the tissues

<sup>6</sup> Pages 257 and 258.

first seek to replenish their store of glycogen before permitting the carbohydrate to be burned. In the diabetic dog the depletion of glycogen is even greater than in fasting. Hence the delayed effect upon the R. Q. of the first dose of Extract 62 (2) given to Dog 8 may be ascribed to the fact that the sugar fed was being converted by the insulin first to glycogen, and it was only after this demand on the part of the tissues had been reasonably satisfied after the second dose of extract and the third dose of sugar, that oxidation could take place rapidly enough to raise the quotient plainly.

The quantitative relationship between extract and sugar is doubtless another factor, upon which more can be said in a later paper. In the earlier experiments on cats<sup>6</sup> and in those about to be reported on dogs there is evidence that the more pancreatic tissue there is represented in the dose the greater is the effect on the respiratory metabolism. The bearing upon this relationship of the abrupt return to the diabetic quotient referred to above and to be noted again below, cannot be definitely decided at this time. The rather obvious inference has already been hinted at; but the matter is complicated by the discovery recently in the extract of a fraction which has the power to act in just the opposite way to insulin; namely, to raise the blood sugar both of normal and diabetic animals enormously. The experiments which support this conclusion and the means of separating this fraction will be reported in another communication. It will suffice here to say that this fraction was probably present in all the extracts described in this paper and may therefore have been a contributing factor in the results obtained with them. If this fraction should raise the blood sugar by checking the combustion of glucose it would have the effect of depressing the quotient. Whether it acts more slowly than insulin proper or must wait for its opportunity until the sugar-burning principle has produced a certain effect would be at this time a matter of pure conjecture.

*Confirmation of Earlier Experimental Results by Stomach Administration.*

Having established beyond a doubt the correctness of the method of extraction previously employed (28) we were interested further to repeat, if might be, the results obtained by administration

<sup>6</sup> Page 256.

of the extract by stomach together with a sufficient amount of alkali to neutralize the gastric juice. Quite aside from any question of specific destructiveness of the gastric juice, it was not to be expected that unconcentrated extracts put into the stomach would be as potent as when given subcutaneously; for nearly all drugs, and particularly organo-therapeutic agents are diminished in their action by the various hazards of the alimentary tract. Murlin and Kramer had given the entire extract from one dog's pancreas, and sometimes of two. It was important to determine what would be the minimal dose for a therapeutic effect from extract made from slaughter-house material. The ineffectiveness of ordinary doses given by stomach without alkali (see Tables III and IV) is probably to be ascribed to destruction of the active principle by pepsin rather than by the acid of gastric juice, for it is now well known that insulin will withstand acidities up to 0.05 N HCl and higher for a considerable time. Hence the use of alkali was not for the purpose of neutralizing the acid *per se*; but for the purpose of inactivating pepsin.

We are confident the experiments about to be described could be extended indefinitely. In only one instance was there a complete failure to raise the quotient and this was doubtless explained by the fact that the extract, originally potent, had been left too acid and had deteriorated with age, for it was subsequently found to be worthless.

The respiratory metabolism in this group of experiments was determined by a closed circuit apparatus comprised of the respiration chamber described in the perfusion papers (26), and a Benedict universal table with a spirometer on a separate stand. This apparatus was chosen merely for greater speed. It was thoroughly tested by means of alcohol checks and found to be satisfactory within 2 or 3 per cent, even without residual analyses. Oxygen was fed in by hand from a cylinder and measured on its way to the chamber by means of a Bohr meter. The chief objection to this form of apparatus with so small a total capacity as 70 liters, which this has, lies in the disturbing effect of a sudden liberation of moisture as by urination or defecation. Without residual analyses<sup>7</sup> it is impossible to compensate for this, and many periods of

<sup>7</sup> In the earlier work residual analyses were always made.

observation had to be discarded for this reason. In none of the experiments reported below was there any difficulty from this cause. Mere restlessness on the part of the animal, if this amounts to nothing more than turning around a few times and provided he is quiet for at least 2 or 3 minutes just before closing the period, does not disturb the respiratory quotient. Panting, of course, would be ruinous and in the experiments reported care was always exercised to keep the temperature of the chamber well below 30°C., the point at which dogs ordinarily begin to pant. Several experiments were lost because the animal was so large that it

TABLE IX.

Dog 9. Pancreatectomy at 4 p.m. Nov. 21, 1922.

Date.	Period.	Time.	CO <sub>2</sub>	O <sub>2</sub>	R.Q.
			liters	liters	
Nov. 25	I	11.50—12.21 p.m.	2.764	3.555	0.777
	II	12.21—12.54 "	2.932	4.054	0.723
		2.40 p.m.		Dog given 300 cc. No. 87 (2) extract unconcentrated + 20 gm. glucose + 0.05 N NaOH.	
	III	5.23—5.52 p.m.	2.688	3.516	0.764
	IV	5.52—6.25 "	3.192	3.952	0.807
Nov. 26	V	6.25—7.07 "	3.777	4.734	0.798
		4 p.m.		Dog died having shown utilization of 31 gm. glucose.	

was impossible to hold the temperature at this level. Corrections on the volume of oxygen for temperature and pressure changes were made (29).

In the first trial (Dog 9, Table IX) the experiment was not so clearly demonstrative as could be wished because the dog did not show, or at least the apparatus did not yield a perfect diabetic quotient to start with. The second period the quotient was 0.72 and knowing from other evidence that the animal was profoundly diabetic we report the experiment in the belief that the first quotient was due to some error unaccounted for.

Preparation 87 (2) had been previously tested for potency and found satisfactory. The unconcentrated extract had been standing in the ice box for several days. It was simply neutralized to approximately 0.01 N HCl and filtered. The 300 cc. dose prepared in this way corresponds to about 70 gm. of the fresh pancreas of beef from which it was made. The highest rise in the quotient came in the 4th hour from time of administration, corresponding well with the lowest blood sugar obtained from a single injection given by vein.

The second trial (Dog 10, Table X) was much more satisfactory in every way. It was only the 3rd day following pancreatectomy and yet the R. Q. was at the level indicative of total intolerance. This time the extract was

TABLE X.  
Dog 10. Pancreatectomy. Nov. 28, 1922.

Date.	Period.	Time.	CO <sub>2</sub>	O <sub>2</sub>	R.Q.
1922			liters	liters	
Dec. 1	I	12.40—1.35 p.m.	2.943	4.271	0.689
	II	1.35—2.47 "	4.073	6.033	0.675
	III	2.47—3.48 "	3.330	4.697	0.708
Dec. 2		3.40 p.m.	Dog given 50 cc. No. 87 (2) concentrated in 300 cc. 0.05 N NaOH + 20 gm. glucose.		
	I	4.53—5.23 p.m.	1.497	1.977	0.757
	II	5.23—6.23 "	2.932	3.682	0.796
	III	6.23—7.41 "	3.966	4.297	0.923
	IV	7.41—8.42 "	3.287	3.183	1.03
	V	8.42—	Dog very restless.		
Dec. 3		11.00 a.m.	D:N shows utilization of 17.3 gm. glucose.		

TABLE XI.  
Dog 10. Pancreatectomy. Nov. 28, 1922.

Date.	Period.	Time.	CO <sub>2</sub>	O <sub>2</sub>	R.Q.
1922			liters	liters	
Dec. 13	I	5.23—5.54 p.m.	0.992	1.466	0.677
	II	5.54—6.29 "	0.993	1.286	} 0.682
	III	6.29—6.59 "	0.845	1.427	
		7.20 p.m.	Dog given 50 cc. No. 93 (2) extract concentrated in 300 cc. 0.55 N NaOH + 20 gm. glucose.		
Dec. 14	IV	9.35—10.07 p.m.	1.242	1.532	0.810
	V	10.07—10.47 "	1.446	1.842	0.785
	VI	10.47—11.19 "	1.094	1.555	0.688
		11.00 a.m.	Dog died. Vomited probably half of solution before being placed in chamber, none thereafter.		

purified and concentrated twenty times so that the 50 cc. dose represented approximately 250 gm. of fresh pancreas. There was an unmistakable increase in the quotient in the 2nd hour following administration, and it

continued to rise as long as the respiratory exchange was followed, reaching the level 1.03 between the 4th and 5th hour. It is quite probable the dog was near the point at which convulsions from an overdose of insulin begin; for he was so restless following this last period that the next period was ruined. The urine collected up to 11 a.m. the next day showed a utilization of 17.3 gm. out of the 20 gm. given at 3.40 p.m., assuming that all of the 20 gm. would have been eliminated in this time if no antidiabetic substance had been given.

The third trial (Dog 10, Table XI) was not so successful as the second because the dog could no longer retain the fluid mixture in the stomach. Nevertheless, the animal, starting off with a diabetic quotient, showed as before a good rise during the fourth period, but this time it was not sustained. Declining from 0.81 to 0.785 in the fifth period, in the sixth period (4 hours) it had fallen all the way back to the diabetic level. Extract 93 (2) used in this experiment had proved unusually potent in tests on blood sugar; hence there is every reason to believe that if the dog could have retained the full 50 cc. dose (equal to 250 gm. of pancreas) the reaction would have been just as positive as in the previous experiment with the same dog.

The necessity for control observations with the sugar and alkali alone is obvious. This had been done many times by Murlin and Kramer in their work on the influence of alkali; but has been repeated in the recent work also several times. Table XII contains one such control entirely typical of those which have been obtained throughout.

This table contains besides the results of several attempts to reduce the effective dose of extract to a point below the equivalent of 250 gm. of fresh pancreas. The 15 cc. dose of concentrated Extract W6B given on Jan. 7 represented approximately half that amount and gave a barely perceptible rise of quotient in the 3rd hour after administration. This continued for 3 hours and then fell off abruptly to the diabetic level. The next day the quotient was found to be about 6 points above the diabetic level and this was repeated again on the morning of Jan. 9 after administration of a large dose of very feeble extract on Jan. 8 (only the preliminary periods of this experiment are reported for the reason that the animal urinated in the second observation period<sup>8</sup>). While these two doses were both so feeble as to produce only a very moderate increase in quotient the level at which this was found next day in each case goes to show that the benefit to the animal was much greater than indicated by the early quotients. Moreover, the utilization of sugar calculated from the D:N ratio in the 24 hour urine (see Remarks column) confirms this. The diet consisted exclusively of meat (ground steak). On Jan. 8 it was found that 13 gm. out of the 20 gm. given on Jan. 7 had been utilized. On Jan. 9 nearly the entire quantity (20 gm.) given on Jan. 8 had been utilized.<sup>9</sup>

<sup>8</sup> See page 292.

<sup>9</sup> These facts make all the more puzzling the sudden drop in the R. Q. to 0.67 and 0.68 after the 5th hour on Jan. 7.

The dose given on Jan. 9 was again equivalent to about 125 gm. of beef pancreas. The rise in quotient above the preliminary level up to the 3rd hour was once more very slight after which it took the usual tumble to

TABLE XII.  
*Influence of Extracts Given by Stomach Tube.*  
Dog 11. Pancreatectomy. Dec. 23, 1922.

Date.	Period.	Time.	CO <sub>2</sub>	O <sub>2</sub>	R.Q.	Remarks.
			liters	liters		
1922 Jan. 6	I	1.00— 2.06 p.m.	4.164	6.177	0.66	Dog not perfectly quiet.
	II	2.06— 3.02 3.15 p.m.	3.737	5.090	0.73	Given 300 cc. 0.05 N NaOH with 20 gm. glucose.
	III	4.18— 5.05 p.m.	2.856	3.885	0.73	Quiet.
	IV	5.05— 5.52	2.790	4.027	0.69	"
Jan. 7		10.45 a.m.				Given 15 cc. concentrated Extract W6B with 20 gm. glucose in 300 cc. 0.05 N NaOH.
	I	12.05—12.50 p.m.	2.606	3.650	0.71	
	II	12.50— 1.53	3.586	4.939	0.73	
	III	1.53— 2.55	3.446	4.512	0.76	
	IV	2.55— 3.55	3.486	4.636	0.74	
	V	3.55— 4.56	3.258	4.853	0.67	
	VI	4.56— 6.13	3.936	5.767	0.68	
Jan. 8	I	11.58—12.33 p.m.	2.077	2.763	0.75	
	II	12.33— 1.00	1.670	2.221	0.75	13 gm. sugar utilized.
Jan. 9	I	12.36— 1.27 p.m.	3.600	4.815	0.75	19.9 gm. sugar utilized.
	II	1.27— 2.22 2.35 p.m.	3.737	5.015	0.75	
						Given 14 cc. concentrated Extract 99 (2) with 20 gm. glucose in 320 cc. 0.05 N NaOH.
	III	3.10— 4.15 p.m.	4.368	5.711	0.76	Somewhat restless.
	IV	4.15— 5.15	4.724	6.048	0.78	
	V	5.15— 5.59	3.111	4.569	0.68	
Jan. 10		2.45 p.m.				50 rabbit units concentrated extract with 20 gm. glucose in 330 cc. 0.05 N NaOH.
	I	3.40— 4.41 p.m.	3.842	5.029	0.76	13 gm. sugar utilized.
	II	4.41— 5.40	3.696	4.837	0.77	
	III	5.40— 6.42	3.833	4.782	0.80	
	IV	6.42— 7.46	3.544	4.402	0.81	

TABLE XII—*Concluded.*

Date.	Period.	Time.	CO <sub>2</sub> liters	O <sub>2</sub> liters	R.Q.	Remarks.
1923						
Jan. 12		11.05 a.m.				Given 15 cc. concentrated Extract 99 (3) with 20 gm. glucose in 300 cc. distilled water.
	I	12.43—1.46 p.m.	4.195	5.478	0.77	
	II	1.46—2.42 "	3.747	4.807	0.78	
	III	2.42—3.47 "	3.996	5.071	0.79	
	IV	3.47—4.55 "	3.874	5.438	0.71	
Jan. 13						26 gm. sugar utilized.

0.68. But, again, the utilization of sugar as calculated next day was 13 gm. out of the 20 gm. given with the extract.

On Jan. 10 a much larger dose of extract was given. Having tested this lot on normal rabbits after the manner introduced by Macleod and his coworkers (30) it was possible to gauge the dose in terms of rabbit units. By the same method of preparation we have recently secured well over 100 rabbit units per 1,000 gm. of beef pancreas, from which it appears that the dose probably represents in the neighborhood of 500 gm. The quotient was not determined previous to administration of the extract in this instance, but was found in the 2nd hour following to be 0.76 from which level it rose gradually to 0.81 in the 5th hour. Unfortunately, the urine the next day was contaminated and the utilization of sugar could not be determined.

After so large an effect on the R. Q. it was deemed necessary to let the animal rest 1 day to give time for this to wear off completely before proceeding with the dosage trials. On Jan. 12, 15 cc. of a very potent preparation, equivalent to approximately 15 rabbit units, were given with the usual accompaniment of sugar, but dissolved in distilled water instead of in 0.05 N alkali. The first three quotients (3rd to 5th hours) showed the usual rise after a moderately effective dose and in the fourth period (6th hour) came the sudden fall, this time to only 0.71. The urine analysis the next day, however, showed the largest utilization of sugar we have yet recorded after stomach administration; namely, 6 gm. more than the amount fed with the extract on Jan. 12. This must have come from the 350 gm. of ground beef steak consumed by the dog during the 24 hours. The experiment indicates clearly that in the depancreatized dog at least (which, of course, has no trypsin to interfere) a moderate dose of concentrated extract can survive the gauntlet of gastric digestion even without the aid of alkali. Naturally, this observation is being followed up at once; but this paper has already been postponed too long and cannot be held for the results. Mention should be made of the fact that Dog 11 lived nearly 40 days as the result almost entirely of the stomach administration and then was allowed to die only because the available extract was needed for human cases.

## SUMMARY.

1. Following the method of extraction employed by Murlin and Kramer in 1913-16, extracts of cat's pancreas were administered to the depancreatized cat intravenously and subcutaneously and the effects in the respiratory quotient were studied.

2. After the appearance of Banting and Best's first paper extracts of pig and ox pancreas were prepared by the same method; namely, extraction in 0.2 N HCl and neutralization, without heating, by means of NaOH. Clear filtrates obtained in this way were tried on depancreatized dogs by several forms of administration and the effects on blood sugar and the D:N ratio studied.

3. Comparisons are made as to the effects from these several methods of administration.

4. Comparison is also made between these aqueous extracts and alcoholic extracts made after the method used by Banting and Best.

5. The effects of boiling and the toxic effects resulting from incomplete destruction of trypsin and from incomplete neutralization of the HCl are discussed. Wholly non-toxic extracts were obtained about July 20, 1922.

6. The effects of these extracts of pig and ox pancreas on the respiratory quotient when given subcutaneously are presented.

7. Copying exactly the method of administration by stomach employed by Murlin and Kramer in 1916, their results were duplicated and the effective minimal dose of (extract of) ox pancreas for this method of administration was worked out. Effects on the respiratory quotient as well as upon the utilization of sugar were studied.

## CONCLUSIONS.

1. Acid aqueous extracts of cat's pancreas raised the respiratory quotient in two different experiments from the diabetic level to 0.82 and 0.87, respectively.

2. Extracts of pig and ox pancreas were found to be about equally effective for reducing the blood sugar and the D:N ratio of depancreatized dogs when given intravenously, subcutaneously, and intraperitoneally.

3. Extracts made in acidulated water were just as effective as those made in acidulated alcohol, allowance being made for concentration.

4. Extremely toxic effects are obtained if the trypsin is not completely destroyed or if the acid is not completely neutralized before administration. Illustrations are given from the autopsy reports for several dogs.

5. Boiling in acid medium for at least 5 minutes does not destroy the principle (insuline, Schafer).

6. Filtering through charcoal and Lloyd's reagent removes a large amount, if not all, the insulin.

7. There are two substances in these aqueous extracts, one of which lowers the blood sugar and the D:N ratio and raises the R. Q. (insulin), the other raises the blood sugar of both normal and depancreatized animals and *possibly* causes an abrupt fall in the respiratory quotient to the diabetic level, after 3 to 5 hours. A more complete report on the latter fraction will be made soon.

8. The abrupt fall in the R. Q. referred to has been observed with considerable regularity. It can scarcely be due to exhaustion of the insulin, for the quotient rises again later.

9. The method of stomach administration referred to above has given positive results almost uniformly. The minimal dose of extract necessary to give a perceptible rise of quotient by this method is equivalent to about 125 gm. of fresh ox pancreas. With double this dose the effect in one experiment was a rapid rise in the R. Q. to over 1.0. Even with the minimal dose necessary to produce a rise of quotient there follows in the course of 24 hours a large utilization of glucose. In one instance where no alkali was used, but only extract and sugar were given by mouth, the utilization of sugar was considerably above the amount ingested as sugar. This portion must have been salvaged from the meat ingested, or from protein of endogenous origin.

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## PLACENTAL TRANSMISSION.

### I. THE CALCIUM AND MAGNESIUM CONTENT OF FETAL AND MATERNAL BLOOD SERUM.

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The experiments reported in this paper were carried out as part of a general project for studying placental interchange, especially as related to the transmission of the inorganic constituents of the blood. The total phosphorus content of fetal and maternal serum, as well as the relative amounts of organic and inorganic phosphorus found in each have also been determined and these results are reported in a separate paper. The general plan of experimentation followed was the same as that used by Slemmons and his collaborators in studying the transmission of the nitrogenous constituents of the blood through the placenta; namely, analysis of blood taken simultaneously from the cord and from the arm vein of the mother at the birth of the child. Since the free admixture of venous and arterial blood in the placental sinuses results in conduction to the fetus through the umbilical vein of blood which is not purely arterial in character, and especially since all the constituents thus far studied by this plan, with one exception, have been found to be present in the same amounts in both the fetal and maternal circulations, there seems no basis for the objection that such blood samples are not comparable because the blood from the umbilical vein is chiefly arterial while that taken from the mother is venous. However, we do take exception to the use of whole blood alone, in such experiments. It is now quite generally conceded that figures for whole blood, unaccompanied by hematocrit readings, may be misleading, especially as the concentration of corpuscles is decidedly greater in fetal than in maternal blood, and that the analysis of serum or plasma gives a much better index of the amounts of the various constituents of the circulating medium.

actually available to the tissues. The addition of neutral sodium citrate in quantities sufficient to prevent clotting was found to diminish the amount of calcium in the plasma, this diminution being proportional to the amount of citrate added. Serum was accordingly employed in all our analyses, care being taken to remove the serum from the clot as soon as possible and to avoid hemolysis. Kramer and Tisdall have shown that the calcium values obtained from ashed plasma and by direct precipitation from serum are practically identical.

The only direct comparison of the calcium content of fetal and maternal blood, which we have been able to find in the literature, is contained in a recent paper by Hess and Matzner (1) and their figures are not really comparable since the maternal blood samples were taken within 48 hours after labor, while the fetal samples were taken from the cord at the time of delivery. Their calcium determinations were made upon serum, but they employed the Lyman nephelometric method, the accuracy of which has been seriously questioned. They conclude that their figures show no significant variations from the normal calcium content of serum, the post-partum maternal samples averaging 9.75 mg. per cent, while the average for the determinations on cord bloods was 10.75 mg. per cent. We have been unable to find any published reports on the magnesium content of maternal and fetal blood, except the results of Howland and Marriott (2), who found 2.7 to 3.5 mg. of Mg per 100 cc. of serum in a few analyses on placental blood.

Although experimental evidence has been lacking for a direct comparison of the fetal and maternal circulations, the reports of several investigators might lead one to suspect the condition disclosed by our experiments; namely, that the calcium content of the serum is generally below normal at the time of delivery, while that of the infant tends to be above the normal range for adults. Kehrer (3) demonstrated a fall in the calcium content of whole blood during the latter half of pregnancy. Meigs, Blatherwick, and Cary (4) did not find any significant lowering of the calcium of whole blood or plasma in cows during the same period. De Wesselow (5), using the direct precipitation method of Kramer and Tisdall, has recently shown that the serum calcium is frequently low in the later months of pregnancy, although his average values are within normal limits, with the possible exception of those for

the 6th and 7th months. In seventeen cases of normal women from 2 to 10 months pregnant, in which plasma (or serum) would appear to have been used for the determination of the inorganic constituents, Krebs and Briggs (6) also noted in a few cases calcium values below normal in the last weeks of pregnancy. In our own studies on the composition of the blood during pregnancy, which will appear in a later paper and which include some 75 observations on women in the course of normal pregnancy, we have found a fairly consistent and appreciable lowering of the serum calcium in the later months of gestation. Some evidence has also been recorded to show that the calcium content of the serum or plasma in the new-born is above the normal range for adults. Jones (7) reported an average of 12.3 mg. of Ca per 100 cc. of plasma in infants between the ages of 4 hours and 12 days. While the absolute values found in these determinations are open to question, because they were done on citrated plasma and by the Lyman method, the results seem to be uniform in indicating a relatively high content of calcium in the plasma of the new-born. Howland and Marriott (2) analyzed a small number of placental bloods and reported figures averaging 10.9 mg. of Ca per 100 cc. of serum. While Hess and Matzner (1) report no individual determinations, it is to be noted that their average figure for cord blood, 10.75 mg. of Ca per 100 cc. of serum, is above the average figure for serum calcium in normal adults (10 mg. per 100 cc.). Meigs, Blatherwick, and Cary (4) also found the plasma calcium high in new-born calves.

#### EXPERIMENTAL.

Calcium determinations were made upon the maternal and fetal serum by the direct precipitation method of Kramer and Tisdall (8). Analyses were done in triplicate and excellent checks were obtained. For the determination of the serum magnesium a combination of the method of Kramer and Tisdall (8) and that of Briggs (9) was employed. The magnesium in an aliquot part of the supernatant fluid from the calcium precipitation was precipitated as ammonium magnesium phosphate according to the directions of Kramer and Tisdall. This precipitate was dissolved in normal sulfuric acid, after thorough washing with ammoniacal alcohol, and the magnesium estimated, as suggested by Briggs, by

means of the degree of color developed by the reaction of the phosphate, held in combination with the magnesium, with hydroquinone and molybdate solutions. The manipulation was adapted so that the whole determination was carried out without transfer of the material from the graduated 15 cc. centrifuge tube in which the precipitation was made. Details of the procedure are given below.

The calcium in a 2 cc. sample of serum was precipitated in a 15 cc. graduated centrifuge tube according to the method of Kramer and Tisdall. After this solution had been made up to 6 cc., mixed, and centrifuged, 5 cc. of the clear supernatant liquid were pipetted into a second graduated 15 cc. centrifuge tube for the magnesium determination. To this were added 1 cc. of acid ammonium phosphate solution (2 per cent) and 2 cc. of concentrated ammonium hydroxide. After rubbing with a glass rod on the inside of the tube, the material was allowed to stand over night. It was then centrifuged, the supernatant liquid poured off, and the precipitate washed three times with ammoniacal alcohol (200 cc. of concentrated  $\text{NH}_4\text{OH}$  to 800 cc. of 95 per cent  $\text{C}_2\text{H}_5\text{OH}$ ), using 12 to 15 cc. of wash solution each time, taking care to secure a good suspension of the precipitate in the wash solution, centrifuging 10 minutes at 2,000 R.P.M., and draining the tube well after each washing. The precipitate was then dissolved in 5 cc. of normal sulfuric acid, 1 cc. of molybdate solution (5 per cent ammonium molybdate in  $\text{N H}_2\text{SO}_4$ ) and 1 cc. of 2 per cent hydroquinone solution were added, and distilled water was added to the 10 cc. mark. The green color developed was read in a Duboscq colorimeter after 5 minutes against a standard simultaneously prepared, of the same dilution, and containing the same amounts of normal sulfuric acid, molybdate, and hydroquinone solutions. Usually 3 cc. of Briggs' standard solution of ammonium magnesium phosphate, equivalent to 0.042 mg. of magnesium, furnished about the right amount of color for comparison with the quantities of serum used. A correction was also made for the blank determination.

#### DISCUSSION.

The results of the calcium and magnesium determinations made upon maternal and fetal blood serum are to be found in the accompanying table (Table I). Other data which may be of interest are also included. Figures for the calcium and magnesium found in normal, non-pregnant women are given in Table II.

The most striking feature of these data is the considerable difference between the maternal and fetal serum calcium. The calcium content of the fetal serum is substantially higher than that of the maternal serum in every case investigated. The difference

ranges from 1 to 2.7 mg. per 100 cc. of serum, with an average difference of 1.8 mg. per cent. We have also observed this difference in serum calcium in favor of the fetus in every one of a previous series of fifteen cases, which suggested the present investigation. We do not consider that this difference in serum calcium is to be explained on the basis of a difference in dilution in the fetal and maternal circulations. The evidence cited below, as well as certain unpublished observations of our own, point to the conclusion that the dilution is almost the same on both sides of the placental barrier, the fetal plasma tending to be even more dilute than that of the mother. The figures of Stander and Tyler (10) show the total ash to be higher and the moisture content to be lower in the plasma of the mother than in that of the fetus at delivery. These authors regard the differences found as insignificant and conclude that the plasma ash is identical in the two bloods, although their figures show differences as high as 33½ per cent in favor of the mother and identical values in only one instance. Büttner (11), and also Zangemeister and Meissl (12) have demonstrated that the molecular concentration of fetal and maternal serum, as measured by the depression of the freezing point, is the same. In view of these facts we believe that the very considerable and consistent difference between the serum calcium of the mother and the fetus, which we have found, deserves emphasis. It seems difficult to conceive of any mechanism by which the large amounts of calcium observed in the fetal serum at birth, amounts in excess of what is usually considered the saturation point, could be maintained in intrauterine life in conformity to the laws of osmosis and diffusion, unless more of the calcium is present in the fetal blood in the non-ionizable form or some compensatory action takes place by means of which other ionizable substances are present in sufficiently larger quantities on the maternal side of the placental barrier to equalize osmotic pressure conditions. The latter hypothesis necessitates the supposition that the placenta possesses powers of selective absorption, which there is at present insufficient evidence to support. It is to be hoped that further experiments may afford some explanation of this condition.

It is interesting to note, moreover, that the few substances which have been shown to be present in higher concentration in the





fetal serum or plasma are those which are in greatest demand for the building of new tissue in the growth of the fetus. Morse (13) found the amino-acid content of fetal plasma higher than that of maternal, while the higher concentrations of calcium and phosphates in fetal serum, demonstrated in this and the following paper, would afford optimum conditions for the development of bony tissue. Givens and Macy (14) have estimated, on the basis of analysis of fetuses from 3 to 8 lunar months of age, that the fetal demand for CaO may average 100 mg. per day for the whole period of pregnancy, and that the demand for calcium is always

TABLE II.

*Calcium and Magnesium Content of Serum of Normal Women Expressed as Mg. of Ca and Mg per 100 Cc.*

Subject No.	Calcium.	Magnesium.
	mg.	mg.
1	9.9	2.2
2	10.4	1.9
3	9.9	2.2
4	10.5	2.7
5	10.5	2.3
6	10.1	2.1
7	10.0	2.3
8	9.8	2.4
9	10.0	
10	10.5	
11	10.6	
12	9.7	
Average.....	10.2	2.3

greatly in excess of that for magnesium. Our data show that magnesium is usually present in the same concentration in fetal and maternal serum, although in a few instances the fetal values are higher.

The large amounts of calcium found in fetal serum are even more striking in view of the tendency toward a dilution of the circulating fluid in the mother during the later months of pregnancy, evidence of which was cited earlier in this paper. This tendency to hydremia is carried over into the fetal circulation as far as most constituents of the blood are concerned, most sub-

stances being present in the fetal serum in amounts equal to or even less than those found in the maternal serum. Thus total plasma and serum proteins have been shown by Zangemeister and Meissl (12), Bauereisen (15), and Landsberg (16) to be lower in pregnant and parturient women than in the non-pregnant, and to be still lower in the fetus at birth. Our own experiments have confirmed these findings. The existence, under these circumstances, in the fetal serum of amounts of calcium, not only substantially above those present in maternal serum, but even in excess of the normal physiological range, seems a remarkable adaptation for the provision of conditions favorable for fetal development.

The data presented in Table I need little further comment. We have observed no constant relationship between the calcium or magnesium content of the serum and the age or parity of the mother. The average figure for serum calcium in the female infants is slightly higher than that in the male infants. The other conditions noted seem to be without influence upon the calcium or magnesium content of the serum except that the higher values for serum calcium, which were found in a few instances, may bear some relationship to the occurrence of postpartum hemorrhage in some of these cases, although higher values for serum calcium were found in cases without the occurrence of marked hemorrhage. The low values obtained for serum calcium in the mother in the great majority of cases, are what might have been expected from the low values previously noted in women during the later months of pregnancy. On the other hand, they furnish evidence that there is no appreciable increase in the concentration of calcium in the plasma during labor, such as claimed by Lamers (17) and suggested by him as an explanation for the increased contractions of the uterus at that time. The figures found for serum calcium in normal, non-pregnant women correspond closely, both as to range and average value, to those found by other investigators.

There are few figures in the literature with regard to the normal range of the magnesium content of the serum. Marriott and Howland (18) give 2.2 to 3.5 mg. of Mg per 100 cc. of serum. Kramer and Tisdall (8) observed values between 2.1 and 2.3 mg. per 100 cc. in six normal adults, while Briggs (9) found 2.2 to 2.5 mg. per 100 cc. in five similar cases. Our figures on eight

normal women range from 1.9 to 2.7 mg., with an average of 2.3 mg. of Mg per 100 cc. of serum. The magnesium content of the maternal serum at the end of labor ranged from 1.4 to 3.2 mg., while that of the child at birth showed a range of 1.4 to 3.4 mg. The magnesium values were practically the same in the fetal and maternal serum in fifteen out of twenty-three cases. In eight cases the fetal serum magnesium was somewhat higher. Average values for magnesium were almost identical in the maternal and fetal serum. No constant relationship between variations in the serum magnesium and alterations in the calcium content of the serum seems to exist. As about 65 per cent of the maternal values are below 2.0 mg. per 100 cc., there seems to be a tendency for the serum magnesium to be lowered, but the results are not uniform enough in this respect to admit of general conclusions.

#### CONCLUSIONS.

1. The calcium content of the blood serum is consistently higher in the fetus at the time of birth than in the mother. The average values found were 9.1 mg. of Ca per 100 cc. in the maternal and 10.9 mg. of Ca per 100 cc. in the fetal serum.
2. The serum calcium of the mother at the end of labor is usually below the physiological range for normal, non-pregnant women, while the average maternal serum calcium is distinctly lower than the average value for normal women.
3. The serum calcium of the child at birth usually exceeds the upper limits of the normal physiological range for adults.
4. The magnesium content of fetal and maternal serum is generally the same, although in a few instances higher values were observed in the fetus.
5. There seems to be a tendency in certain cases for both the maternal and fetal serum magnesium to be below normal, although the average values found at the end of labor (maternal, 2.0 mg. and fetal, 2.1 mg. of Mg per 100 cc. of serum) are within the normal range.

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## PLACENTAL TRANSMISSION.

### II. THE VARIOUS PHOSPHORIC ACID COMPOUNDS IN MATERNAL AND FETAL SERUM.\*

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In continuance of the study of fetal metabolism, on the basis of the analysis of simultaneously collected samples of maternal and fetal blood, the various phosphoric acid compounds of the serum have been determined according to the methods of Bloor (1). The general technique employed was that outlined in a previous communication (2), and the analytical procedures were carried out in duplicate without essential modification. All specimens were analyzed immediately after collection in order to avoid any possible errors due to delay in beginning the determinations.

We have been unable to find any equally complete data on maternal and fetal blood, although there are certain statements by other investigators which indicate in general the variations which might have been expected. Thus, McKellips, De Young, and Bloor (3) have shown that, in the plasma of normal young infants (from 2 days to 4 weeks of age): "The organic phosphorus is regularly much higher in the infant than in the adult, resulting also in a higher acid-soluble fraction. Lipoid phosphorus is much lower throughout in the infant than in the adult, . . . ."

Tisdall (4) and Howland and Kramer (5) record the inorganic phosphorus in normal infant sera, and the former says: "The serum of normal infants has a much higher inorganic phosphorus content than is present in normal adults." He found 4.6 to 6.4 (average 5.4) mg. of phosphorus per 100 cc. of serum.

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Hess and Matzner (6) have reported the inorganic phosphorus of the whole blood from the cords of twenty-one normal infants, and have compared the values thus obtained with those of the mothers' blood obtained "a day or two following labor." The average fetal content was 3.71 mg., while the maternal was only 2.89 mg. of phosphorus per 100 cc. They conclude that "inorganic phosphate is considerably higher in the blood of the foetus than in that of its mother," and, that "although the content of inorganic phosphate is higher in the foetal than in the maternal blood, it is markedly lower than that of the young infant." The use of whole blood instead of plasma or serum, and the fact that the maternal specimens were not obtained at the same time as the fetal, detract somewhat from the value of the results.

Meigs, Blatherwick, and Cary (7) investigated the variations of the phosphoric acid compounds in pregnant and lactating cows and in new-born calves and came to several definite conclusions. (a) ". . . toward the end of pregnancy the phosphorus content of the blood and plasma is likely to be reduced." This holds for the total phosphorus, but is more constant in the inorganic fraction. (b) "The lipoid phosphorus in the plasma of new born calves is very low, and increases gradually up to the age of about a year. The inorganic phosphorus of the plasma is fairly high in the new born calves, but tends to increase for some time and reaches a maximum at about the age of 6 months." (c) During lactation the lipoid phosphorus increases, whereas the inorganic phosphorus follows no regular course.

Slemons and Stander (8) determined the lecithin in the whole blood and plasma of simultaneously collected specimens of maternal and fetal blood by the Bloor method and found that this phosphorized fat (lipoid phosphorus) is increased in amount above normal at the end of pregnancy, and that the maternal whole blood or plasma always contains more than does the fetal. When considered in connection with the fact that the mother's blood and plasma both contain considerably more total fat than does the infant's, this finding leads these authors to conclude that "fetal fat. . . . must be synthesised; probably from glucose, which is freely supplied by the mother in accord with the demands of her offspring." Lecithin in the maternal plasma varied from 210 to 280 mg. per 100 cc. (average 241 mg.) and in

the fetal plasma from 190 to 240 mg. per 100 cc. (average 221 mg.). The difference was always in favor of the mother except in one instance where the values were equal; the greatest difference was 80 mg. per 100 cc.

Krebs and Briggs (9) have recently recorded the inorganic phosphorus in the blood of seventeen normal pregnant women from the 8th to the 40th week of gestation. The reported values vary from 1.08 to 2.69 mg. of inorganic phosphorus per 100 cc., and are said not to be different from normal non-pregnant values obtained by the method employed. Although it is not definitely stated anywhere in their article, it is assumed that whole blood was used and that the results are in milligrams of phosphorus per 100 cc.

Table I presents our experimental results in detail. It is apparent that the maternal blood serum is higher in total and lipoid phosphorus; whereas the fetal serum is higher in inorganic and organic phosphorus and, consequently, in their sum, the total acid-soluble phosphorus. These facts appear not only in the average figures but, likewise, in each individual case, except that, in two specimens, the organic phosphorus fraction is slightly higher in the maternal sample.

Table II shows the inorganic phosphoric acid expressed as elemental phosphorus, and is introduced merely to facilitate comparison with the findings of other authors who utilize this method of expression.

Table III gives the lipoid phosphoric acid in terms of lecithin, in which form it is sometimes expressed.

Table IV is introduced to facilitate comparison of our maternal and fetal values with the established normals for women and for infants, obtained with the same analytical procedure. Bloor's figures (10) and those of McKellips, De Young, and Bloor (3) are for plasma instead of serum, but it is assumed on the basis of the statement of Howland and Kramer (5) that there is no essential difference between the two fluids.

At the end of labor, the total phosphoric acid of the serum is increased above normal, largely by reason of an increase in the lipoid phosphorus fraction, for, at this time, the inorganic phosphoric acid is appreciably lowered, while the organic phosphorus varies within normal limits. The new-born child, on the other

## Placental Transmission. II

TABLE I.  
*Phosphorus in Fetal and Maternal Serum Expressed as Mg. of  $H_3PO_4$  per 100 Cc.*

Case No.	Hospital No.	Age.	No. of previous deliveries.	Previous deliveries.	Total.		Lipoid.		Acid-soluble.		Inorganic.		Organic.		Anesthesia.		Delivery.		Weight. g.	Height. cm.	Weight. g.m.
					Materinal.	Fetal.	Materinal.	Fetal.	Materinal.	Fetal.	Materinal.	Fetal.	Materinal.	Fetal.	Materinal.	Fetal.	Obstetrical CHCl <sub>3</sub> .	Spontaneous.			
1	11,092	20	1	39.3	29.9	28.1	10.5	10.4	17.7	11.5	17.8	0.1	0.1	0.1	0.1	Obstetrical	Spontaneous.	F.	3,210	3,210	
2	11,099	18	0	76.2	37	352.1	10.1	12.1	20.8	8.1	10.9	4.0	9.9	0.1	0.1	“	“	M.	2,790	2,790	
3	11,116	19	0	54.0	47	845.9	21.0	12.7	15.8	9.0	12.2	3.7	3.6	0.1	0.1	“	“	F.	3,470	3,470	
4	11,130	19	0	69.4	35	771.4*	27.0*	11.8	17.6	9.5	14.2	3.2	3.4	0.1	0.1	“	“	M.	2,880	2,880	
5	11,133	18	0	73.5	33	640.3	10.6	12.0	21.0	11.7	17.6	0.3	3.4	0.1	0.1	“	“	F.	3,100	3,100	
6	11,137	28	0	46.4	34	957.9*	23.7*	11.1	16.2	7.9	13.5	3.2	2.7	0.1	0.1	Surgical	Low forceps.	M.	2,930	2,930	
7	11,148	19	0	57.1	29	1,389.9	10.2	12.8	17.3	11.9	13.8	0.9	3.5	0.1	0.1	“	“	F.	3,080	3,080	
8	11,149	28	0	49.8	26	737.4	11.5	9.0	13.6	9.6	12.5	1.1	1.1	0.1	0.1	Obstetrical	Spontaneous.	“	2,500	2,500	
9	11,155	18	1	67.7	32	2,36.4	13.6	12.2	21.8	9.3	16.5	2.9	5.3	0.1	0.1	“	“	M.	2,930	2,930	
10	11,161	30	2	58.3	41	732.2	16.0	10.4	22.7	9.1	19.1	1.3	3.6	0.1	0.1	“	“	“	3,180	3,180	
11	11,163	18	0	49.5	26	734.9	22.0*	12.2	16.7	10.0	10.7	2.2	6.0	0.1	0.1	“	“	“	“	“	
Average.....				58.3	34.1	38.5	12.9	11.5	18.3	9.8	14.4	2.3	3.9								

\* Not included in computing averages.

TABLE II.

Inorganic  $\text{H}_2\text{PO}_4$  expressed as P.  
 $(\frac{\text{H}_2\text{PO}_4}{3.2})$

Case No.	Maternal.	Fetal.
	mg. per 100 cc.	mg. per 100 cc.
1	3.6	5.6
2	2.5	3.4
3	2.8	3.8
4	3.0	4.4
5	3.7	5.5
6	2.5	4.2
7	3.7	4.3
8	3.0	3.9
9	2.9	5.2
10	2.8	6.2
11	3.1	3.3
Average .....	3.1	4.5

TABLE III.

Lipoid  $\text{H}_2\text{PO}_4$  expressed as lecithin.  
 $(\text{H}_2\text{PO}_4 \times 8.0)$

Case No.	Maternal.	Fetal.
	mg. per 100 cc.	mg. per 100 cc.
1	225	84
2	417	81
3	367	168
4	571*	216*
5	322	85
6	463*	190*
7	311	82
8	299	92
9	291	109
10	258	128
11	279	176*
Average .....	308	104

\* Not included in averages.

hand, as compared with the 2 to 14 day old infants studied by McKellips, De Young, and Bloor (3), has a higher total phosphoric acid and an increased acid-soluble fraction, whereas the lipoid phosphorus is slightly lower. The sex and birth weight of the infant apparently have no influence upon the blood phosphorus, although the size of our series is too small to permit of general conclusions in this regard.

#### DISCUSSION.

*Lipoid Phosphoric Acid (Lecithin).*—Our results confirm those of Slemmons and Stander (8) that, at the time of delivery, the fetal blood has less lecithin than has the maternal. They, however,

TABLE IV.

Phosphorus expressed as mg. of $\text{H}_3\text{PO}_4$ per 100 cc.	Normal women. (Plasma) accord- ing to Bloor.		Maternal. (Serum) accord- ing to author.		Fetal. (Serum) accord- ing to author.		Normal infants (2 to 14 days). (Plasma) accord- ing to McKellips, De Young, and Bloor.	
	Extremes.	Average.	Extremes.	Average.	Extremes.	Average.	Extremes.	Average.
Total .....	31.0-41.0	36.2	39.3-76.2	58.3	26.7-47.8	34.1	18.5-43.0	29.7
Lipoid .....	19.0-29.0	24.9	28.1-52.1	38.5	10.1-21.0	12.9	10.1-21.4	14.8
Acid-soluble....	9.4-14.3	12.4	9.0-12.8	11.5	13.6-22.7	18.3	8.8-25.0	16.5
Inorganic .....	8.0-13.8	11.2	7.9-11.9	9.8	10.7-19.1	14.4	3.7-13.9	9.6
Organic .....	0.0- 4.0	1.3	0.0- 4.0	2.3	0.1- 9.9	3.9	1.3-11.4	7.0

Table arranged to show comparison of our figures with those of Bloor (10) for normal non-pregnant women and with those of McKellips, DeYoung, and Bloor for young infants.

detected relatively slight differences, and we can offer nothing to explain why they never found more than a 25 per cent variation, while the maternal values which we report are from two to four times as great as the fetal. Our figures are the more striking and, if subsequently confirmed, will greatly reinforce the contention of Slemmons and Stander that fetal fat must be synthesized from the glucose which passes the placental barrier by diffusion.

The increased lipoid phosphorus in the mother's serum at the time of delivery, likewise confirms the findings of Slemmons and Stander (8) although here again the differences which we detected

were greater than theirs. Meigs, Blatherwick, and Cary (7) in their work on pregnant cows demonstrated a lowered lipoid phosphorus toward the end of pregnancy, with a sudden rise immediately after delivery and at the onset of lactation. It would seem that these contradictory findings may be explainable only on a basis of a difference of species. Although Slemmons and Stander (8) relate the increased lipoid phosphorus in the blood serum at the time of labor, to a preliminary change in the organism looking toward a successful lactation, it is only by a study of this substance during pregnancy and in early lactation that these changes can be followed completely.

*Inorganic Phosphoric Acid.*—The observations of Hess and Matzner (6) that the fetal blood contains more inorganic phosphorus than does the maternal blood, are confirmed. We are, however, unable to substantiate their findings that the fetal blood contains less inorganic phosphoric acid than the blood of young infants shortly after birth. In fact, our values—10.7 to 19.1 mg. of  $H_3PO_4$  per 100 cc.—are so much higher than those reported for young infants by McKellips, De Young, and Bloor (3) that we are inclined to believe that there is a rapid fall in the inorganic phosphorus content of infant's serum shortly after birth.

Tisdall and Harris (11) have emphasized the fact that the apparent optimum for inorganic phosphorus is about 5.4 mg. of phosphorus per 100 cc. and the recognized optimum for calcium is about 10 mg. per 100 cc. One of us (2) has recently demonstrated that fetal serum contains well over 10 mg. of calcium per 100 cc., even though the maternal values are considerably below normal, and it is here shown that the inorganic phosphate values at least approach the optimum figure. The fact that our subjects were drawn from the poorer classes, in whom the progress of intrauterine ossification is apparently somewhat less rapid than in the better nourished groups of our population, may account for the below optimum quantities. It is, nevertheless, quite evident from the uniformly higher values in the new-born children, that there must be in operation some natural mechanism for providing the fetus with quantities of the chief bone-producing substances—calcium and phosphates—most conducive to rapid skeletal growth.

This increase of inorganic phosphorus on the fetal side of the placental barrier is not dependent upon a concentration of the

fetal serum, but must be explained in some other way. The arguments supporting this contention have been given previously (2) in the discussion as to the explanation of the higher calcium values in fetal blood.

The low maternal inorganic phosphate values are quite comparable to the subnormal calcium content of the serum obtaining at the time of delivery. In all probability this is largely a dilution phenomenon, since there is a demonstrable hydremia during the latter months of pregnancy. Definite data on this subject will shortly be published.

Inorganic phosphorus thus becomes the third substance to be demonstrated constantly in higher concentration in the plasma or serum on the fetal side of the placenta; the other two are the amino-acids (12) and calcium (2). It is extremely interesting that these substances are all absolutely essential to the synthesis of the various organs and structures of the fetal body, whereas the materials which have been demonstrated in equal concentration—urea, uric acid, creatinine, and creatine—are waste products. At any rate, the diffusion hypothesis, advocated by Slemmons and his school (13), can scarcely be used to explain the proven inequalities. On the other hand, the finding of these essential building materials in higher concentration in the fetal serum, is not a valid argument, in itself, for the idea that the syncytial cells of the placenta have a selective activity, whereby the growing fetus receives an optimum quantity of the supplies it most needs. At present it is perhaps difficult to advance any other explanation, but it is at least conceivable that the whole phenomenon may be governed entirely by known physicochemical laws.

The total phosphoric acid figures vary with the lipoid and inorganic values, and, therefore, need no comment. The higher values for organic phosphorus in the fetal serum are of interest even though their significance is unknown. McKellips, DeYoung, and Bloor (3) noted especially high values in the infants they studied, and it is possible that these unknown compounds may be associated with growth.

#### SUMMARY.

The distribution of the various phosphoric acid compounds between the maternal and fetal sera, separated by the barrier

of placental cells, has been studied by Bloor's method. Evidence has been adduced in support of the belief that the fats and lipoids are synthesized in the fetal organism, and it has been demonstrated that the inorganic phosphoric acid furnishes another example of an easily diffusible body being maintained in higher concentration in the fetal blood, where an optimum quantity is desirable for satisfactory bone production.

#### CONCLUSIONS.

1. Lipoid phosphoric acid is increased above normal in the blood sera of women at the time of delivery, whereas it is present in small quantities only in the fetal blood.
2. Inorganic phosphoric acid is diminished in the serum of normal women at the end of parturition, probably as a result of physiological hydremia or hydroplasmia occurring at that time, while the fetal serum contains quantities comparable to the high values obtaining in young infants and assumed to represent the optimum for bone construction.
3. By reason of the greatly increased quantity of lipoid phosphorus present, the total phosphoric acid is increased at the end of labor and is considerably higher in the maternal serum than in the fetal.
4. Organic phosphoric acid tends to be somewhat higher in the serum of the fetus than in the mother.

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## THE ACIDOSIS OF ETHER ANESTHESIA IN THE DOG.

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In summarizing the results of previous workers and of their own experiments, Van Slyke, Austin, and Cullen (1) concluded from the consistent fall of both the blood pH and the alkaline reserve that a true acidosis occurs in ether anesthesia, due either to the introduction of acid into the blood, or to the withdrawal of base from it. On the basis of hyperventilation experiments, Henderson and Haggard (2) believed that such changes in the acid-base equilibrium of blood under ether could be explained as a compensatory reaction to an acapnial process. Van Slyke and his coworkers were of the opinion that the evidence indicates otherwise.

Although our experiments were made in 1920, with technique not as advanced as that in force at present, we believe our data may be sufficiently well interpreted in the light of present conceptions of the acid-base equilibrium to lend support to the contentions of Van Slyke, Austin, and Cullen (1), and more importantly, to indicate that the acidosis of ether anesthesia is due to the withdrawal of base from the blood rather than to the entrance of acid into it.

### EXPERIMENTAL PROCEDURES.

We observed the course of ether anesthesia in dogs with respect to the following: (a) The respiratory minute volume as an index of total lung ventilation, using the body plethysmograph previously described (3). (b) The alkaline reserve of arterial blood plasma, using the method of Van Slyke (4). (c) The pH of whole arterial blood, using an electrometric method, with the hydrogen electrode for blood determinations devised by McClendon (5), and the improved calomel electrode introduced by Koehler (6).

(d) The total acetone bodies of whole arterial blood, using the method of Van Slyke and Fitz (7).

We obtained results on fourteen dogs, the observations on which fall into two groups: (1) A series of eight animals, in which a tracheotomy was performed, and in which the depth of anesthesia was controlled by regulating the passage of the air inspired through a Wolff bottle containing ether. Etherization was prolonged in this series for 6 hours, when the animals were killed. (2) A series of six dogs, in which etherization was accomplished by drop administration upon a gauze mask held over the animal's nostrils, and in which as soon as the so called third stage of anesthesia was reached the ether was withdrawn, permitting prompt recovery.

In all cases blood was drawn directly from the femoral artery into a syringe, through the intact skin, in amounts not exceeding 15 cc. at a time. It was immediately transferred under oil, out of contact with air (except that used for acetone body determinations), to tubes containing just enough saturated potassium oxalate solution to prevent coagulation. In the oxalated plasma for the alkaline reserve measurements, obtained by centrifuging, slight hemolysis occurred in blood from three animals after the prolonged administration of ether. All determinations were made at once, and in the case of most of the pH and alkaline reserve in duplicate. The total amount of blood drawn from any one animal was not in excess of 60 cc., which in dogs averaging over 9 kilos in weight may be considered negligible. Blood was obtained immediately before etherization in all cases, and in the first series at the 1st or 3rd hours after etherization was begun and at the 6th hour, while in the second series, it was taken as soon as the third stage of anesthesia was reached, 30 minutes after ether was withdrawn, and finally, in most cases, 12 hours after. Respiratory tracings were made continuously except during the brief intervals when blood was being drawn. There was no struggling except in the animals recovering from etherization.

We were fortunate enough in the first series to obtain five cases where respiration was depressed below normal for the entire duration of the experiment, except for about 15 minutes when etherization was first commenced. Results for a typical case of this kind may be seen in Table I, while the data for the five are averaged and summarized in Table II. In all these animals

the pH and alkaline reserve values fell markedly as etherization progressed, while the acetone bodies did not vary much beyond the limits of error. Where considerable changes in the acetone bodies did take place, it was inconstant, in some instances tending to

TABLE I.  
*Effects of Prolonged Ether Anesthesia in Dog 4, Weight 8.7 Kilos.*

Time.	Respirations per min.	Lung ventilation.	Per respiration.	Pulse.	Alkaline reserve.	pH	Acetone bodies.	Remarks.
a.m.		cc. per min.	cc.		vol. per cent		mg. per l.	
10.43	16	2, 102	131	100	49.6	7.62	38.4	Lipemia.
10.57		Etherization and tracheotomy.						
11.10	32	2, 059	64	160	24.6	7.53		Lipemia.
12.00	24	1, 705	71	110				
p.m.								
1.00	20	1, 768	88	100				Slight lipemia, some hemolysis.
2.00	20	1, 520	76	110	24.6	7.28	35.8	
3.00	20	1, 387	69	78				
5.00	20	1, 289	64	72	19.0	7.18	82.5	No lipemia, slight hemolysis. Killed.

Total amount of blood drawn was 54 cc.

TABLE II.  
*Average Effects of Prolonged Ether Anesthesia in Five Dogs, in Which Respiration Was Uniformly Depressed. Average Weight 9.1 Kilos.*

Time after starting ether.	Lung ventilation.	Alkaline reserve.	pH	Acetone bodies.
hrs.	cc. per min.	vol. per cent		mg. per l.
0	2, 101	46.8	7.59	40.6
½	2, 152			
1	1, 928	30.2	7.39	44.2
3	1, 363	24.5	7.27	35.8
4	1, 412			
6	1, 197	21.5	7.19	51.2

fall slightly, and in others showing an inclination to rise. In no case in our ether experimentation did we observe anything comparable to the changes occurring in the amounts of acetone bodies noted under morphine narcosis, and previously reported upon (3).

In the other three animals of this first series, we maintained a

light anesthesia throughout, with consequent increase in lung ventilation above normal, and except that the acetone bodies tended uniformly to decrease, we observed the same effects as noted when etherization was deep, and respiration suppressed.

TABLE III.  
*Effects of Prolonged Ether Anesthesia in Dog 1, Weight 12.1 Kilos.*

Time.	Respirations per min.	Lung ventilation.	Per respiration.	Alkaline reserve.	pH	Acetone bodies.	Remarks.
a.m.		cc. per min.	cc.	vol. per cent		mg. per l.	
9.48	20	2,840	142	46.6	7.60	56.5	
10.03		Etherization and tracheotomy.					
10.15	72	6,408	89	29.2	7.43		Salivating freely.
11.00	36	4,276	118	31.0	7.43		Plasma volume diminished.
p.m.							
1.00	24	3,673	153	27.6	7.31	32.8	
2.00	68	4,148	61				
3.00	28	4,233	151				
4.00	24	6,069	252	20.3	7.24	20.4	Killed.

Total amount of blood drawn was 58 cc.

TABLE IV.  
*Average Effects of Prolonged Ether Anesthesia in Three Dogs, in Which Respiration Was Uniformly Increased. Average Weight 10.3 Kilos.*

Time after starting ether.	Lung ventilation.	Alkaline reserve.	pH	Acetone bodies.
hrs.	cc. per min.	vol. per cent		mg. per l.
0	2,054	47.9	7.60	49.0
½	5,661			
1	4,648	32.4	7.37	
3	4,003	26.7	7.26	20.7
4	4,865			
6	5,388	21.4	7.22	15.4

Results for a typical experiment are shown in Table III, while the averaged summary for the three dogs may be found in Table IV.

In the animals of the second series, by the time the third stage of anesthesia was reached, usually within 5 minutes after etherization was begun, we noted a fall in the alkaline reserve of about 10 volumes per cent, and a drop in the blood pH of from 0.2 to 0.3 points, while the acetone bodies showed a distinct rise. At this

moment the lung ventilation was usually still considerably above normal. 30 minutes after the withdrawal of ether, the lung ventilation was in some instances markedly above normal, while in others it had fallen to a normal level or even slightly below. However, in all cases at this moment, a slight increase in the pH and alkaline reserve figures was found above that observed when the third stage of anesthesia had been reached. The acetone bodies in some cases still showed an upward trend, while in others they had fallen. 12 hours after the withdrawal of ether, with respiration generally somewhat above normal, the pH and alkaline reserve were also higher than before etherization, whereas the acetone bodies were lower. The data are averaged and summarized in Table V.

TABLE V.  
*Average Effects of Short Etherization in Six Dogs. Average Weight 9.8 Kilos.*

Time.	Lung ven-ti-lation. cc. per min.	Alkaline reservc. vol. per cent	pH	Acetone bodies. mg. per l.
0	3,322	45.5	7.58	63.1
5 min. after starting ether.....	6,272	35.1	7.35	106.7
30 min. after withdrawing ether.....	6,424	38.7	7.40	125.3
12 hrs.    "    "    " .....	3,733	47.6	7.61	57.6

#### DISCUSSION.

Our figures show no relation between the blood reaction and respiratory activity in ether anesthesia, except perhaps in the first 5 minutes of etherization, when overventilation may be a factor in lowering the blood alkali. But when this effect is compared with data obtained for both suppressed and increased respiration over a 6 hour period under ether, it must be considered very small indeed. On this evidence we see no reason for believing that the blood changes under ether are compensatory for a supposed acapnia, as stated by Henderson and Haggard (2), and as admitted as a possibility by Collip (8).

We found no increase in acetone bodies which could conceivably account for the acidosis of ether anesthesia. Other acid products may be formed, of course, but this is unlikely, since the acetone bodies seem to be the typical non-volatile acids produced in disturbed metabolic conditions, especially when any interference with oxidative processes takes place.

The alternative explanation, as indicated by Van Slyke, Austin, and Cullen (1), is the withdrawal of base from the blood. In this connection, the figures reported by Stehle, Bourne, and Barbour (9) are of interest. These authors discovered that although during ether anesthesia the excretion of sodium and potassium in the urine was diminished—probably because of the ether anuria, as suggested by MacNider (10)—there was a marked increase in the excretion of these alkalies in the postanesthetic period, so that the total daily amount was considerably above normal. On clinical grounds, Reimann (11) also inclines somewhat toward this explanation.

#### SUMMARY.

In prolonged ether anesthesia in dogs, whether respiration is steadfastly diminished or increased, the pH and alkaline reserve of blood fall markedly, while the acetone bodies show no significant change.

In very short ether anesthesia in dogs, in which hyperventilation takes place, the pH and alkaline reserve of blood fall, and the acetone bodies tend to rise. These tendencies are reversed 30 minutes after the withdrawal of ether, and 12 hours later the pH and alkaline reserve are slightly above normal values, and the acetone bodies below.

The evidence indicates that the acidosis of ether anesthesia can be explained neither by a compensatory reaction to an acapnia, nor by the entrance of acid into the blood, but rather by the withdrawal of base from it.

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## FAT-SOLUBLE VITAMINE.

### XI. STORAGE OF THE FAT-SOLUBLE VITAMINE.\*

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Even a cursory examination of the published data of investigations dealing with the occurrence of the fat-soluble vitamine<sup>1</sup> reveals the fact that there is a great lack of uniformity in the amount of growth obtained when rats are placed on various diets low in their content of this dietary essential. This might well be explained by difference in vitamine content of the diets,

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

<sup>1</sup> In this paper are presented data dealing with the ability of the animal to store those vitamines which are soluble in fats. No distinction is made nor is any attention paid to what this storage may in reality represent as this is futile until the distinction between the fat-soluble vitamines which may exist and the effects they produce are better understood. We have paid particular attention in making our observations to the maintenance of growth and the incidence of ophthalmic and respiratory symptoms.

There has been accumulated considerable evidence by Mellanby, Hess, McCollum, and ourselves that vitamine A is distinct from the antirachitic vitamine although Mellanby came to a different conclusion and Hess was not sure whether he was dealing with another vitamine or some other factor. If it should be finally concluded that the antirachitic property is resident in another vitamine, our data may later be interpretable from this standpoint. At present we prefer to speak of the vitamine concerned as a fat-soluble vitamine as the solubility in fats is a property which divides the vitamines into two large groups. This inclusive consideration in no way detracts from the significance of the observed facts.

We shall later present data obtained in experiments with dogs and rats which at present support the thesis that it is more correct to speak of the fat-soluble vitamines than vitamine. If such a conclusion should ultimately be warranted, then we shall later use this nomenclature distinguishing in this group between vitamine A and the antirachitic vitamine. Until we have concluded our experiments we shall use the old terms.

one diet containing a more satisfactory content of other vitamines, known and unknown, than the other, but this is immediately excluded by the fact that these variations occur in the same experimental groups. Another explanation which might be proposed is that some individuals are possessed, through hereditary influence, of either a greater assimilating capacity for deficient amount of vitamine or of an ability to meet the growth impulse with smaller amounts of this substance. From what is known of the variability of those physiological processes which deal with growth this also does not appear tenable, especially in colonies which have been closely inbred for many years. Such variations as do occur can usually be explained by incidence of disease or some unappreciated dietary factor and, furthermore, the variations in growth of the rats on the vitamine deficient diets are frequently of such an order as to be entirely outside of the range of variations attributable to difference in unlimited growth impulse.

Osborne and Mendel (1) in 1921 in analyzing the factors which might be responsible for the divergent conclusions reached in different laboratories in studies of the vitamine content of lard discuss the probable factors concerned. They paid especial attention to the fact that the rations so called fat-soluble vitamine-free might not be entirely vitamine-free; but apparently they did not come to any very positive conclusion for they write as follows:

"It has been alleged that the ability of animals to grow for some time in the supposed absence of fat-soluble vitamine is due to 'exceptional vitality' of the individuals or to reserve stores of the vitamine in the body. Unless we are to accept some indefinite explanation of this sort, it seems necessary to conclude, in the light of our experience, that removal of the fat-soluble vitamine from even purified proteins and carbohydrates is accomplished with far greater difficulty than has been hitherto suspected. An entirely convincing crucial experiment, in which nutritive failure immediately ensues upon the administration of diets fully adequate *in every respect* except for the presence of fat-soluble vitamine, remains to be made."

In part we are inclined to agree with Osborne and Mendel for certainly the fat-soluble vitamine cannot be readily extracted from casein with hot alcohol which has been extensively used for this purpose and which is probably its best solvent. We

have, however, been frequently impressed with the necessity of giving consideration to the storage factor in spite of the fact that for the water-soluble vitamine this is a negligible factor (2).

Storage of the fat-soluble vitamine during times of plenty to enable the animal to meet temporary deficiencies in the diet has been pointed to by a number of investigators.

Sherman and Smith (3) state:

*"That vitamin A may be stored to an important extent in the body is indicated not only by the results of feeding body fats and glandular organs as sole sources of this vitamin in the diet, but also by the fact that when growing animals previously well fed are placed upon diets certainly deficient in vitamin A and often apparently devoid of it, they usually continue to grow for some time, often doubling their body weights before signs of nutritive deficiency appear."*

They present some unpublished data of Sherman and Boynton who fed rats three diets composed of wheat and dried whole milk where the milk as the source of fat-soluble vitamine made up, respectively, one-sixth, one-third, and two-thirds of the ration. After being reared on this diet and then placed on a diet deficient in fat-soluble vitamine, growth was in proportion to the amount of milk previously fed.

Evans and Bishop (4) arrived at similar conclusions making use not of growth increments but of the disturbance of the oestrous cycle in the rat which they found to be related to fat-soluble vitamine deficiency. They state:

*"When adults instead of having an excellent nutritive past history have had to exist for 80 days on a casein-cornstarch-lard ration with only 2% butter fat and when they now have all butter fat withdrawn, they develop the sign of vitamine A deficiency on the average in 68 instead of the 100 days required when the diet before butter withdrawal was identical but contained 9% butter fat. When animals have had a still poorer nutritive past and have been reared from the time of weaning on the casein-cornstarch-lard 2 % butter regime, the withdrawal of butter may give our vitamine A deficiency sign within 4 days, always does so by the 44th day and usually within 14 days."*

We have for some years given consideration to the storage of the fat-soluble vitamine as the most important factor in determining the growth of our rats when put on a diet deficient in this vitamine, and accordingly have sought to minimize it as

much as possible without interfering with reproduction and rearing of the young. The necessity for such precautions can be readily seen. When young rats are started immediately after weaning on rations of unknown vitamine content for determination of the same, the stored vitamine will function in an additive capacity with the vitamine of the ration and growth will result in proportion to the total. This has sometimes made it advisable to keep the rats after weaning on a ration free from fat-soluble vitamine, allowing them to stop growing entirely, before putting them on the ration of which the vitamine content was to be determined. In practice this does not always work out successfully. Some rats will have stored so much of the vitamine, previous to weaning, that growth will be continued until most of the growth impetus has been expended; change of diet to a vitamine-containing ration then results in minimal response. Sometimes after almost normal growth the rats will fail precipitously due to respiratory infections before change of ration can be made. In our experience such infections are far more liable to occur in the near mature than in the very young, and when prevalent, make results incident to dietary change of uncertain value.

These difficulties in experimentation we have succeeded in removing entirely by keeping all of our stock rats on a standard ration which is approximately constant in fat-soluble vitamine content and by maintaining accurate record of their age. If the rats do not weigh at least 40 gm. at 23 days of age they are not used for fat-soluble vitamine work.

#### EXPERIMENTAL.

Some of our first data on the importance of vitamine storage in determining the future life history of the rat were obtained from experiments outlined for another purpose. A group of four rats had been fed a ration of white corn 30, rolled oats 30, wheat bread 30, dried beef muscle 8.5, calcium carbonate 1, sodium chloride 0.5, and 5 cc. of whole milk per rat daily. On this ration one of the females reproduced and raised a litter of five young, but contracted an ophthalmia which practically blinded her and persisted to the end of the experiment. The young were raised to an average weight of 50 gm. in 27 days

which is but slightly below normal. We are at a loss to account for the fact that the young were raised when the mother was afflicted with an ophthalmia, as it has been our general experience that young usually fail to live under these conditions. It is possible that the ophthalmia here was due to dietary factors other than deficiency of the fat-soluble vitamine, but this we believe improbable as the ration fed is well known not to carry a superabundance of the vitamine and, furthermore, this rat as well as two other rats on this ration contracted respiratory infection in severe degree; in one case this was the immediate cause of death.

At the time that the young were in condition to be weaned we desired to make a comparison of the efficacy of various methods of treatment used to free commercial casein from fat-soluble vitamine. As we were short of animals raised on our standard stock ration and as these young were in excellent condition we departed from our customary procedure of using only stock animals and distributed these among various experimental groups. They were used in experiments designed to bring out the effect of heat treatment, according to Drummond and Coward (5), on the fat-soluble vitamine content of casein. It so happened contrary to our expectations that the crude commercial casein on which the experiments were based was already practically free from the vitamine so all the caseins, heated and unheated, were substantially alike. As the other constituents of the rations in the different groups were identical the variability of the ration factor was non-existent. The ration was composed of casein 18, agar 2, salts 32, 4 (6), yeast 2, and alcohol-extracted dextrin 74.

The relations observed in the performances of the animals are brought out in Chart I. Both groups averaged 50 gm. in weight and were started at almost the same age, those taken from the experimental colony being 27 days old while those taken from the stock colony were 24 days old. While the animals were almost on a parity with respect to age and weight their behavior is seen to be conspicuously different. In the first place the rats reared on the ration deficient in fat-soluble vitamine reached a final weight of approximately only one-half that of the stock animals and in the second place they all became afflicted with an ophthalmia in from 3 to 4 weeks time. Of the stock rats

only one contracted an ophthalmia and that occurred after the group had been on the ration 7 weeks. These results were so suggestive that we decided to carry out an extended series of experiments to bring out the importance of the storage factor.

In the first series a comparison was made in the growth, well being, and viability of young rats when put on a diet deficient in the fat-soluble vitamine after having been reared on an ordinary ration in contrast with some reared on a ration abundantly supplied with this vitamine.

The ordinary ration was our usual stock ration VIII composed of yellow corn 76, linseed oil meal 16, crude casein 5, alfalfa 2,

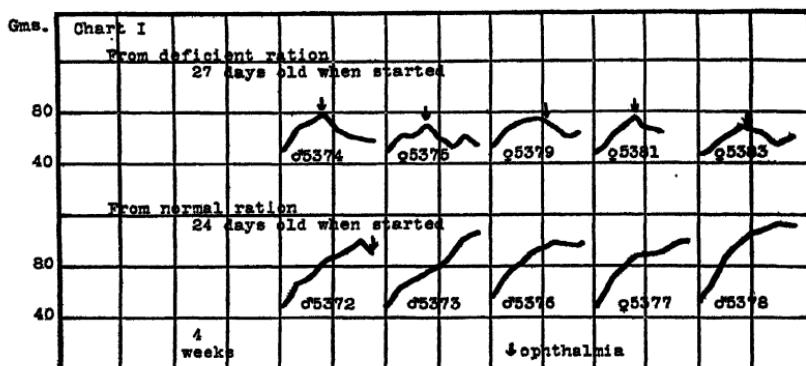


CHART I. Shows the difference in resultant growth and incidence of ophthalmia dependent upon the fat-soluble vitamine content of the previous ration on which the rats had been raised. The deficient ration was composed of white corn 30, rolled oats 30, wheat bread 30, dried beef muscle 8.5, calcium carbonate 1, sodium chloride 0.5, and 5 cc. of whole milk per rat daily. The fat-soluble vitamine sufficient ration was composed of yellow corn 72, oil meal 15, alfalfa 2, crude casein 5, calcium carbonate 0.5, sodium chloride 0.5, and whole milk *ad libitum*. The experimental ration to which the rats were changed was composed of casein 18, agar 2, salts 32, 4, yeast 2, alcohol-extracted dextrin 74. The casein was not always the same; some of it was crude casein, some was casein that had been heated for 24 hours at 98°, and some had been treated for 1 week at 98° exposed in thin layers according to the method of Drummond. The uniform performance of the animals within the groups shows that the vitamine content of the casein was the same. Rats 5374 and 5375 were on casein which had been heated 1 week, Rat 5379 on casein heated for 24 hours, and Rats 5381 and 5383 on the crude casein. In the other series, Rats 5372 and 5373 were on casein heated for 1 week, and Rats 5376, 5377, and 5378 on casein heated for 24 hours.

sodium chloride 0.5, calcium carbonate 0.5, and whole milk *ad libitum*. We have used this ration for years in raising rats for fat-soluble vitamine experiments and, therefore, have knowledge of what results could be expected with a reasonable degree of certainty. The fat-soluble vitamine-rich ration was composed of the same constituents with 5 parts of cod liver oil added to 95 of the other ingredients. It was fed to the nursing females beginning 4 days after birth of their young and later was also accessible to the young until they were put upon the experimental ration.

The experimental ration was composed of casein 18, salts 32, 4, yeast 2, agar 2, and dextrin 74. The casein was a commercial casein very finely ground, hot alcohol-extracted. In extracting casein with hot alcohol it has a very decided tendency to pack in the extractor making percolation impossible. To obviate this it was mixed with an equal weight of dextrin. The extraction was continued for 1 week, with two changes of hot alcohol daily. It was then heated to drive off the alcohol, dried at 70-80° for a few days, and ground. The bulk of the dextrin in the ration was not extracted.

The females used for the production of the young were all young females in good condition. Their litters shortly after birth were reduced to six young to insure normal rearing of the same. From each litter four animals were used for each experimental group. This enabled us to check up on variations in storage of vitamine liable to occur with rats as kept in our stock colony.

On the experimental ration the young were kept in groups of four in cages 2 feet square and 20 inches high with pine shavings for bedding. They were fed and given water daily and the cages also cleaned daily to reduce consumption of feces. If this is not done it would have been entirely possible that greater longevity subsequent to a vitamine-rich diet might have been due to consumption of excreta formed from the previous diet, as on deficient diets rats sooner or later become coprophagists (7).

All experiments were continued until death of the animals. In the meantime they were weighed weekly and notations on their conditions made as found necessary. The incidence of

edema and inflammation of the eyes and respiratory disturbances as indicated by rhinitis, sneezing, coughing, and dyspnea was especially noted.

The results obtained are shown in Charts II and III; the former showing the data obtained from the rats raised on an ordinary ration and the latter on a ration very rich in fat-soluble vitamine. First of all there is seen a very pronounced difference

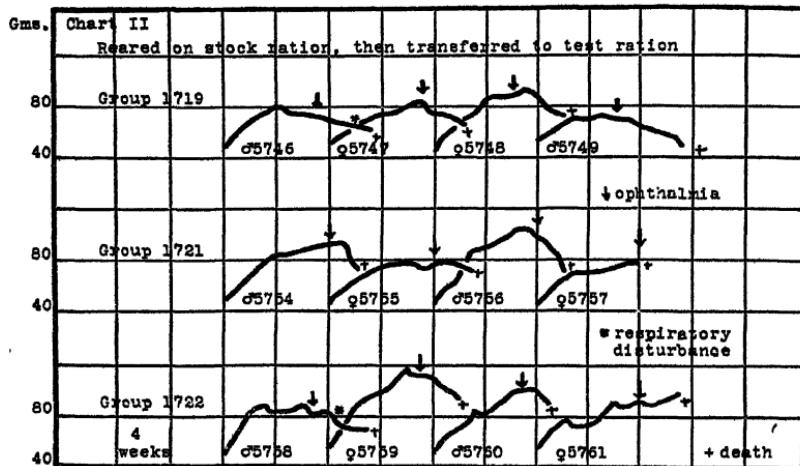


CHART II. Shows the growth obtained in young rats when put on a ration practically free from fat-soluble vitamine after having been reared on our standard stock ration. Each group represents four animals taken from separate litters of six. Groups 1719 and 1721 were started at an age of 22 days, Group 1722 at 25 days. Of the twelve rats all developed ophthalmias in from 6 to 8 weeks, average 7, after being put on the ration. Respiratory infections were not so evident, only two cases developing, in Rat 5758 at 10 weeks and in Rat 5746 at 8 weeks after being put on the ration. The infections were indicated by a hemorrhagic rhinitis. Death occurred in from 8 to 11 weeks, average 10.

in growth though the animals were all started at about the same size and age with an average of 50 gm. at 24 days in Chart II as compared with 56 gm. at 22 days for those shown in Chart III. The former averaged 88 gm. as the maximum weight, while the latter averaged 176 gm. Maintenance of life varied in the same direction, an average of 10 weeks in Chart II as compared with 15 weeks for those shown in Chart III. Difference in the time

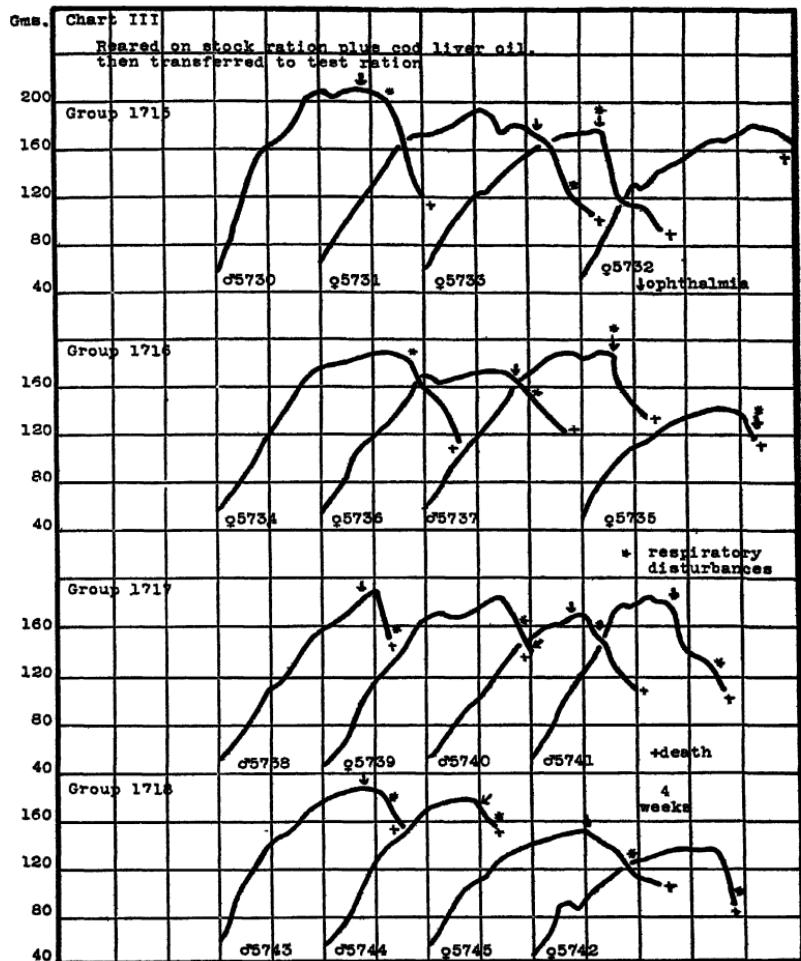


CHART III. Shows the growth obtained in young rats when put on a ration practically free from the fat-soluble vitamine after having been reared on our standard stock ration which had been enriched in its fat-soluble vitamine content by the addition of 5 per cent of cod liver oil. Each group represents separate litters, the first one containing only four individuals the second five, and the last two, six. They were started at approximately the same age; Groups 1715 and 1716 at 22 days, and Groups 1717 and 1718 at 21 days.

Of the sixteen rats only two, *viz.* Rats 5734 and 5742, did not develop an ophthalmia, the others all developed it in from 11 to 17 weeks, average 13, after being put on the ration.

One rat did not show any signs of respiratory disease, the others contracted it in from 13 to 19 weeks, average 14, after the feeding of the fat-soluble vitamine-free ration was begun.

Death of the animals terminated the various feeding trials in from 13 to 21 weeks, average 15 weeks.

before ophthalmia made its appearance is also notable, an average of 7 weeks for those shown in Chart II as compared with 13 weeks in Chart III if we omit from consideration Rats 5734 and 5742 which did not show any optical symptoms. The incidence of respiratory trouble in the two lots is hardly comparable as only two cases, respectively, at 8 and 10 weeks were observed in the animals from the ordinary ration. These were indicated as a rhinitis. In the other lot all except one, *viz.* Rat 5732, developed pulmonary infections after being, on the average, 14 weeks on the experimental ration.

In the second series of experiments an attempt was made to study the effect of feeding a ration very high in fat-soluble vitamine for 1 and 2 weeks, respectively, after weaning and before the change to a fat-soluble vitamine-low ration was made. The fat-soluble vitamine-rich ration was the same as that fed in the previous series, but in the fat-soluble vitamine-low ration all the dextrin was alcohol-extracted and the casein had been extracted with very dilute acetic acid as described (7), before being extracted with alcohol.

The results show conclusively the rapidity with which the rat can store the fat-soluble vitamine. In 1 week's time Group 1663 (Chart IV) consumed 214 gm. of the ration representing 10.7 gm. of cod liver oil which according to test gave the rats an intake of fat-soluble vitamine six times as large as required for normal growth. Even if the ration had not been enriched with cod liver oil an additional week on the stock ration alone would have meant continued normal growth for at least 2 to 3 weeks subsequently.

The above fact is of considerable importance in animal husbandry practice where attempts have been made to demonstrate with pigs a difference in nutritive value of white and yellow corn variously supplemented. Invariably the mistake has been made to pay little attention to the age of the pigs when selected for the experiments. As long as they weighed from 50 to 60 lbs. and appeared to be in a thrifty condition they were supposed to be suitable for such experiments. It is not surprising from this that some have found it impossible to demonstrate a difference in the ability of yellow corn as compared with white corn to support growth. We venture to assert that if the experi-

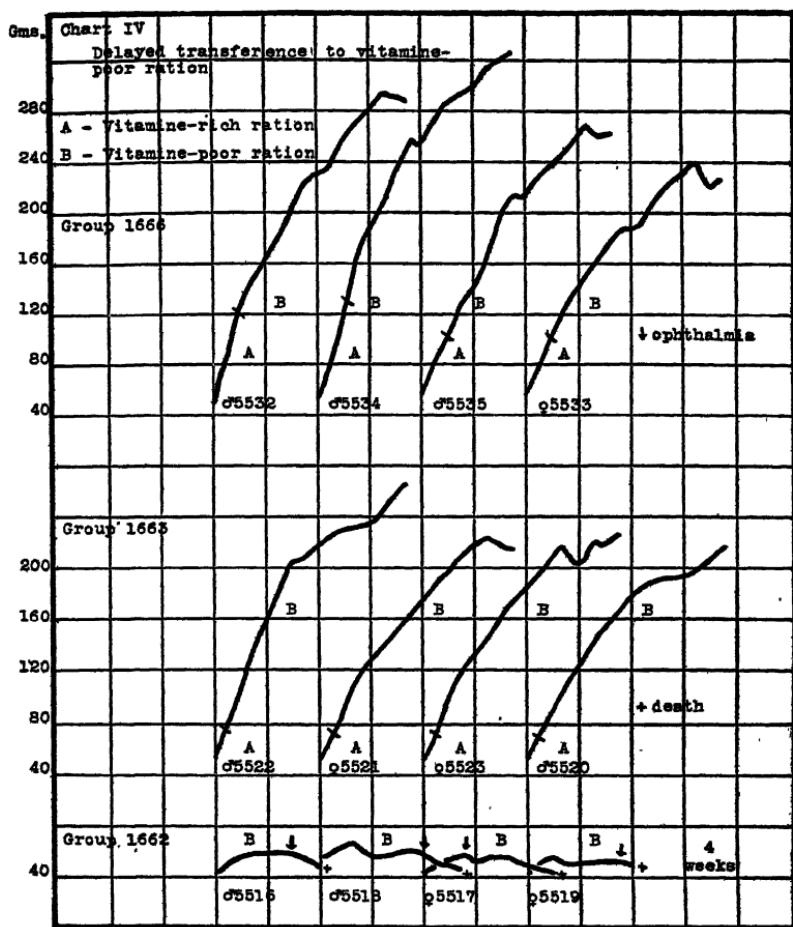


CHART IV. Shows the effect of starting young rats directly after weaning (Group 1662) on a fat-soluble vitamine-free ration in comparison with others fed for 1 week (Group 1663) and for 2 weeks (Group 1666), respectively, before the change, on a ration rich in the vitamine. The rats were taken from four litters, each group representing one rat from each litter. They varied in age from 21 to 22 days.

After the change, in spite of the natural decrease in growth impetus with age, Group 1662 gained on the average 4 gm., Group 1663, 127 gm., and Group 1666, 135 gm. in the following 8 week period.

In Group 1662 all the rats developed an ophthalmia within 8 weeks and died within 11 weeks after the experiment was started; the members of the other groups remained in normal condition as long as the experiment was continued.

menters had used the animals at an early enough age before much storage had taken place no difficulty would have been encountered. It is obvious that weight alone is no criterion of suitability as the rate of growth may be slightly depressed and yet if the ration is sufficiently rich in the fat-soluble vitamine, storage of it may occur. In our rat work we have found an age variation of 2 to 3 days of moment and rats which reach a normal weight at 28 days of age instead of the usual 23 days are now no longer used for our fat-soluble vitamine experiments.

Another application of these facts is possible in human physiology. Clinicians have frequently commented on the fact that outside of the reports of Mori (8) and Bloch (9) few, if any, authentic cases of ophthalmia due to vitamine deficiency have been reported. Hess and Unger (10), especially, have presented concrete evidence showing the lack of symptoms in a number of children receiving a diet of skimmed milk for months. Here undoubtedly two factors were operative: In the first place skimmed milk is not free from this dietary essential and in the second place considerable amounts may have been drawn from stored reserves.

#### *Fat-Soluble Vitamine in Rat Livers.*

Osborne and Mendel (11) demonstrated that pig's liver is an excellent source of the fat-soluble vitamine, which fact has been confirmed by us in numerous experiments. As to the presence of this vitamine in other tissues some demonstrations are available. Both McCollum and Davis (12) and Osborne and Mendel (13) found it in pig's kidney and pig's heart although in this latter organ it undoubtedly is not present in abundance. In animal fats such as beef fat, whale oil, and oils extracted from internal organs such as the liver its presence is well recognized. *A priori* it does not seem admissible that the vitamine is distributed in these tissues because of its indispensable rôle in these various organs. It seemed far more logical to assume especially in view of its association with the fats that excess of the vitamine is stored as such in various organs for future use.

We have put this matter to experimental test using rat livers as the material for investigation. So far as we know no data on the fat-soluble vitamine content of rat livers are available,

but we assumed that ordinarily their content of this dietary essential is comparable with that of pig's liver and this when fed at a level of 1 per cent of our ration had been found to be on the border-line of furnishing enough of this vitamine. If storage of the vitamine takes place we would naturally expect more of the vitamine to be found in the livers of normal rats than in those

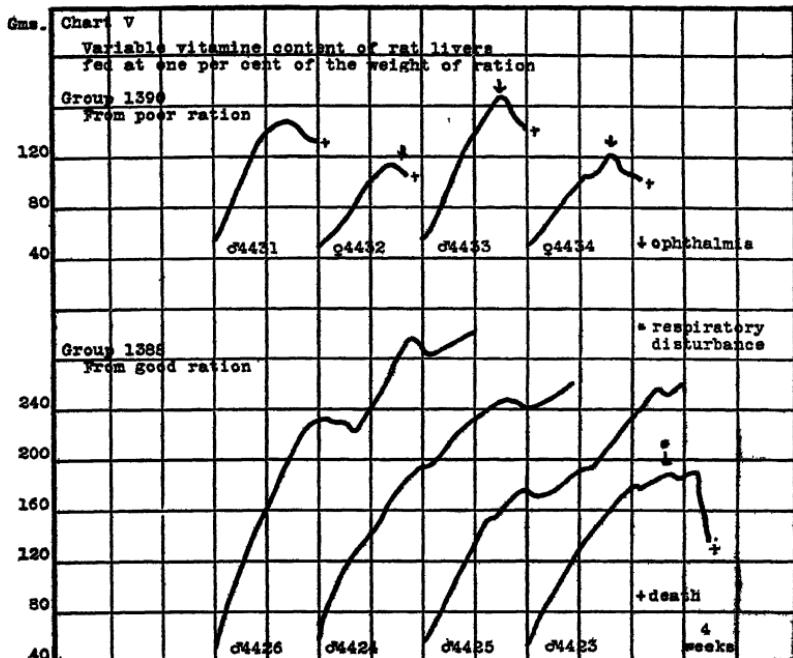


CHART V. Shows the difference in growth of young rats on rations deriving their fat-soluble vitamine from rat livers obtained from rats reared on rations low in fat-soluble vitamine as compared with those reared in rations of normal fat-soluble vitamine content. The rats in Group 1390 receiving the former reached a maximum weight of 115 to 172 gm. then failed rapidly and died within a period of 10 weeks after the experiment was started. Rats 4432, 4433, and 4434 all contracted ophthalmia before death. By way of contrast, rats in Group 1388 reached a maximum weight of 188 to 297 gm., continued growth at a normal rate to the end with no signs of ophthalmia, and with one exception, Rat 4423, lived till the termination of the experiment. Apparently 1 per cent of normal liver does not, however, provide an excess of fat-soluble vitamine because Rat 4423 in the 14th week on the ration contracted ophthalmia and respiratory infections.

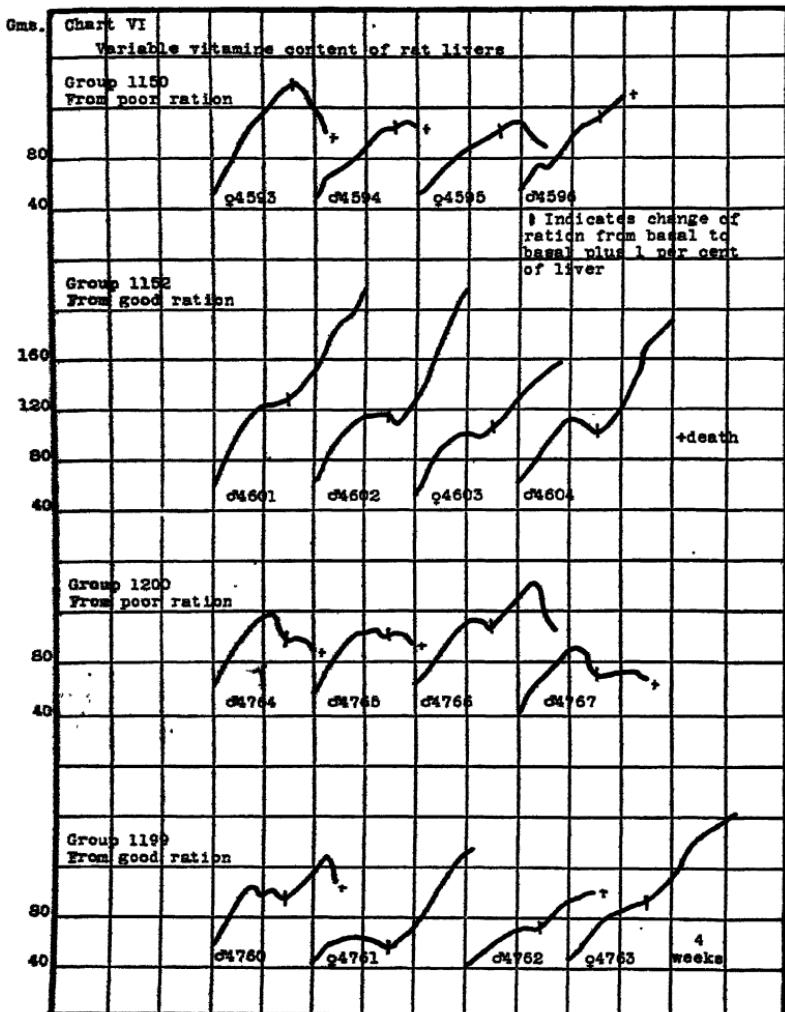


CHART VI. By first inducing failure by feeding a ration low in fat-soluble vitamine, it was possible to secure more pronounced recovery with the liver of rats previously on normal rations than with those on fat-soluble vitamine deficient rations. Experiments proving this were conducted in two series.

In the first there are included Groups 1150 and 1152. In Group 1150 all the individuals contracted ophthalmia and none improved upon change of ration. In Group 1152 likewise all had contracted ophthalmia approximately at the time of cessation of growth. With change of the ration all started to grow rapidly, the eyes of Rats 4601, 4603, and 4604 became normal and only one, *viz.* Rat 4602, continued to show slight signs of infection in spite of its rapid growth.

In the second series Groups 1200 and 1199 were used experimentally in the same way. Here all the rats except Rat 4763 contracted ophthalmia without improvement upon change of ration. Although renewed growth was more pronounced in Group 1199 than Group 1200; the livers of these normal rats apparently were not so abundantly supplied with the fat-soluble vitamine as those used in the previous series as indicated by the decreased growth rate as well as by the failure to cure completely the ophthalmias. Rat 4763 remained normal throughout the experiment.

Recovery experiments are always open to criticism in that no complete measure of the degree of failure can be obtained. Often the physiological disturbances are more deep seated than indicated by external symptoms and naturally responses cannot be expected to be uniform.

showing pathological evidence of a deficiency. Accordingly, young rats as soon as they gave pronounced signs of failure on a fat-soluble vitamine deficient diet were etherized, eviscerated, and the livers dried at 95-98°. After thorough drying they were ground to a powder and incorporated in a basal ration of casein 18, agar 2, salts 32, 4, yeast 2, and dextrin 73 to the extent of 1 per cent. The casein had not been alcohol-extracted, neither had the dextrin, but as the same ingredients were used in all the rations the results are comparable. The animals were kept in groups of four in cages 2 feet square provided with shavings for bedding.

In Chart V is shown the difference in growth obtained with young rats when started immediately after weaning on the liver-containing rations, Lot 1390 receiving liver taken from rats previously on a ration low in fat-soluble vitamine and Lot 1388 livers from rats on our stock ration.

Chart VI brings out the same relations in duplicate with a different technique, the rats being allowed to fail on a ration of white corn 40, casein 14, salts 32, 3, salts 35, 1 (6), and dextrin 42 before being changed to the liver-containing rations.

#### SUMMARY.

There is no question but that the rat can store the fat-soluble vitamine in large amounts in its tissues for future use. This storage factor is of considerable moment in experiments where growth of the rat is used as a criterion of the relative amount of the vitamine present. It is of course necessary to have a sufficiency of this vitamine present in the ration of the stock animals to make normal reproduction and rearing of the young possible, but when a large excess is present there is introduced the danger of providing the young with an excess of the vitamine enabling them to grow for a long period of time at the normal rate when put on a fat-soluble vitamine deficient diet.

Even when rats are raised on a ration of optimum fat-soluble vitamine content, it is imperative to start the young on the experimental rations at an early age to minimize storage. It is recommended that for experimental work of this nature the age and weight of the young and the stock ration used should be standardized relative to one another. The practice of feeding

stock rats on a ration variable in character such as garbage or of "throwing in" adjuvants to the usual ration from time to time is to be condemned.

The liver appears to be an important center of fat-soluble vitamine storage, varying in its content of this substance with the ration fed.

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## FAT-SOLUBLE VITAMINE.

### XII. THE FAT-SOLUBLE VITAMINE CONTENT OF MILLETS.\*

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From time to time McCollum has called attention to his observations that millet seed contained more of the fat-soluble vitamine than most of the common seeds used as food for man or beast. In 1917 he makes the statement:<sup>1</sup> "When considered in the light of our former experience with maize, wheat, and oats, all of which have been shown to carry an inadequate amount of the fat-soluble A, it becomes evident that the flaxseed meal and millet seed differ from maize, wheat, and oats in containing a much greater amount of this dietary factor."

Also he<sup>2</sup> says: "It is interesting to note that the content of fat-soluble A is highest in those seeds which are smallest (flax and millet). It seems not unlikely that this may in some measure be related to the relatively large proportion of germ as compared with endosperm in such seeds."

Again he may be quoted (2): "It is of particular interest therefore to compare the effects of flaxseed and millet seed as sources of the fat-soluble A. Both of these are richer in this substance than are the cereal grains, and millet seed proves to be unique among the seeds we have examined as a good source of this dietary factor. When 25 per cent of millet seed was combined with purified foodstuffs, growth was completed and one rat has produced two litters of young. After eight months on this diet she appears to be in perfect nutrition."

In 1919 McCollum stated:<sup>3</sup> "The oil seeds judging from the limited data available seem to contain more of the fat-soluble A than do the cereal grains but less than millet seed. The latter is richer in this substance than any other seed yet studied."

Again,<sup>4</sup> "The list of seed examined included,—wheat, corn, rice, rolled

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<sup>1</sup> McCollum, Simmonds, and Pitz (1), p. 15.

<sup>2</sup> McCollum, Simmonds, and Pitz (1), p. 18.

<sup>3</sup> McCollum (3), p. 179.

<sup>4</sup> McCollum (3), p. 38.

oats, rye, barley, kaffir corn, millet seed, flaxseed, pea and both the navy and the soy bean.—All are with the exception of millet seed below the optimum in their content of the dietary factor, fat-soluble A."

Later McCollum and Simmonds (4) observed that a ration carrying 33 per cent of its weight as millet seed was unable to maintain adult rats. After 60 days confinement all the rats in the group became blind. It is significant that this ration besides the millet contained salts, agar, and dextrin, but no additional protein. The authors state that when the protein content of the diet is satisfactory rats do not go blind when the amount of fat-soluble A contained in 25 per cent of millet is furnished.

We pointed out in 1921 (5) that our experience with millet did not warrant drawing the conclusions that McCollum had drawn yet we surmised that millets might vary in their content of this dietary essential just as we had found variations in the case of different varieties of Indian corn and peas. We are, however, convinced that the early observations of McCollum were in error because we found no significant amounts of fat-soluble vitamine in this same sample of millet or in subsequent tests of the same variety. We are inclined to believe that the fat-soluble vitamine which he found to be present was introduced with the casein. This we believe to be true for two reasons. In the first place the instance where McCollum observed a deficiency of the fat-soluble vitamine occurred as stated where casein had been omitted from the ration and in the second place casein is not readily purified from fat-soluble vitamine by his method of treatment which consists in washing with dilute acetic acid and then drying. There are, however, certain commercial caseins on the market, for instance, the Argentine caseins, which are remarkably low in fat-soluble vitamine content. This is probably due to the fact that these caseins are dried in the open air in the sunlight as it is well known that the vitamine is easily destroyed in the presence of air and light. Another factor which enters into these early results is that the reserves of fat-soluble vitamine function in an additive capacity with the vitamine content of the ration. A young rat well supplied with stored vitamine will grow normally for months on a ration free from it (6). Most of the early investigations including our own on fat-soluble vitamine content of various materials must be evaluated anew with these reservations in mind.

In our experience millets have been found to differ considerably in their content of the fat-soluble vitamine. The variation is of

such degree that it is no longer justifiable to speak of millets as being rich or poor in this constituent unless the variety is mentioned.

#### EXPERIMENTAL.

The data justifying the conclusion that millets show no uniformity in fat-soluble vitamine content were obtained in two series of experiments where failure of growth and incidence of ophthalmia in rats were used as the criteria.

The rats were started on the millet rations at 23 to 26 days of age weighing at that time from 40 to 58 gm. They were fed four in a group, in a cage 2 feet square and 20 inches high, and were bedded with pine shavings. The ration was fed *ad libitum* and fresh distilled water was given daily. The animals were weighed weekly.

Four different varieties of millet seed were used; *viz.*, Common millet, Hog millet, White Wonder millet, and Japanese millet. In size, Common millet was the smallest and Hog millet the largest. In color, Common and Hog millet differed but slightly, both having a light yellow tinge. White Wonder had a distinct yellow color. Japanese millet as fed was for the most part still enveloped in hulls. When freed from these the seeds were of a light pearl grey with a tinge of green. All samples were ground to a fine powder, first in a burr mill and then in a ball mill.

In the first series the rats were fed 40 parts of millet in a ration of casein 14, salts 32 (7), 4, and dextrin 42. The casein was a commercial casein purified as usual (8) in our laboratory and then thoroughly extracted with hot alcohol. The dextrin represented corn-starch partly dextrinized (7) and then hot alcohol-extracted.

The results of these trials are shown in Chart I. Fed at a 40 per cent level in a ration otherwise complete except for fat-soluble vitamine the millets were unable to support normal growth; ophthalmias became incident and frequently infections of the respiratory organs made their appearance.

Of the four millets examined, the Japanese millet, by the prolonged though subnormal growth of the animals receiving it, gave evidence of the presence of appreciable amounts of vitamine. The evidence with the other varieties is not entirely one-sided. Failure of growth speaks against the presence of vitamine, yet our

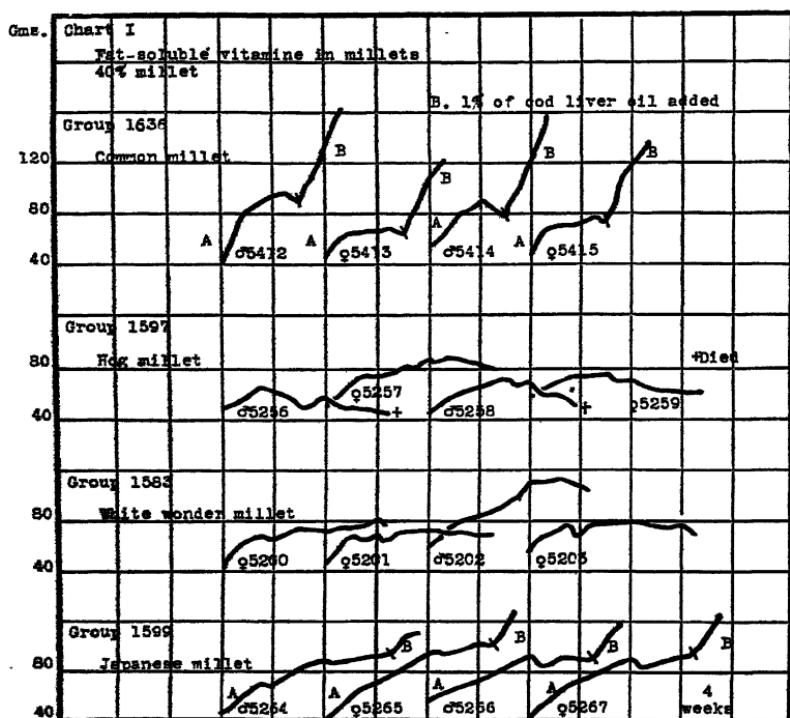


CHART I. 40 per cent of the ration as millet representing the sole source of fat-soluble vitamine is unable to furnish enough of this substance for growth. One variety, however, a variety known as Japanese millet supported growth for a longer time than any of the others. This points to a greater content of this dietary essential in this millet than in the others. That the cause of the ultimate failure of growth was due to a deficiency of fat-soluble vitamine is supported by the resumption of growth which occurred when cod liver oil was incorporated in the rations of Groups 1636 and 1599.

In Group 1636, Rat 5412 showed incipient signs of an ophthalmia at the 6th week. These disappeared with change of ration. In Group 1597, Rat 5256 showed similar signs the 11th week. They did not increase in severity, but during the 13th week the rat contracted rhinitis and died. In Group 1583, Rat 5200 contracted ophthalmia during the 5th week and Rats 5201, 5202, and 5203 during the 10th week. In Group 1599, Rat 5266 showed a slight swelling of the lids of the left eye after the 2nd week on the ration; this promptly cleared up in a few days and probably was due to trauma and not related to the ration.

In Group 1636, Rat 5414 was observed to walk with a peculiar gait suggesting weakness of the pelvic arch. This disappeared after cod liver oil had been fed for 2 weeks.

experience on purified rations usually has been that growth of the magnitude seen here shortly results in failure with the incidence of ophthalmia in 5 to 7 weeks. Here ophthalmias were observed in six animals; in one case in the 5th week, one in the 6th, three in the 10th, and one in the 11th. Most of these were delayed too long for results expected from a ration entirely free from the fat-soluble vitamine.

In the second series the millets were fed at a level of 84 per cent supplemented with 12 parts of casein and 4 parts of salts 32. The casein was alcohol-extracted as before.

In this series, unequivocal evidence of a difference in fat-soluble vitamine content was obtained as seen in Chart II. None of the four varieties tested contained enough for continued well being, the Japanese variety being the richest and the White Wonder next in order. Hog millet and Common millet gave little, if any, better results as far as growth is concerned when fed at the present level of 84 per cent than when fed previously at the 40 per cent level. This might again be taken as evidence indicating entire lack of fat-soluble vitamine. Examined for incidence of ophthalmia, however, none of the rats became infected during the 13 weeks that the Common millet was given. Furthermore, only three cases of respiratory infections were observed, two a rhinitis during the 12th week on both millets, and one a lung infection on the Common millet.

The absence of significant growth on the Hog and Common millets, and the delayed incidence or entire absence of ophthalmia in the 13 weeks of experimentation appears to justify inspection of the data from another point of view. Here the most suggestive theory would be that the fat-soluble vitamine component may actually consist of two entities, vitamine A and the antirachitic vitamine, either one by its absence preventing growth; the one directly preventing the infections such as the ophthalmias and the other abnormal bone formation in rickets as McCollum, Simmonds, and Becker (9) have suggested. In our studies we have no direct evidence of interference with normal bone formation. All our evidence is indirect as we have observed repeatedly the abnormal gait of the animals, as described in the legends of Charts I and II, which we have come to look upon as evidence of rickets. Of these animals, however, no histological examinations were made.

CHART II. 84 per cent of the ration as millet, the maximum that can be incorporated in a ration, is insufficient for maintenance of life over a long period of time. In one case, however, that of Group 1600 on Japanese millet, normal growth was obtained in three out of the four individuals for approximately 4 months before failure supervened. Up to the 19th week no pathological signs of failure were observed but by the 23rd week all had died from infections of the respiratory organs. Ophthalmias were not observed.

In Group 1582 on Common millet, Rats 5196 and 5198 contracted respiratory infections during the 13th week; here also there was an absence of eye infections. Previous to the observation of signs of infection at the 9th to 11th week, abnormal locomotion with the hind quarters was noticed in Rat 5198, and Rat 5196 resented being handled by crying as though in severe pain.

In Group 1598 on Hog millet, Rats 5261, 5262, and 5263 walked abnormally as early as the 8th week, the last mentioned dragging its hind legs and crying as though in pain. At this time none of the rats showed signs of an ophthalmia though this developed in Rats 5262 and 5263 by the 12th week.

In Rat 5260 the eyes, by the end of the 10th week, looked slightly edematous, but our suspicions of the presence of an ophthalmia were not confirmed before death ensued. Rat 5261 by the 12th week contracted rhinitis.

In Group 1584 on White Wonder millet, Rats 5205, 5206, and 5207 all contracted respiratory infections from the 14th to the 15th week. No ophthalmias nor abnormal locomotion were observed.

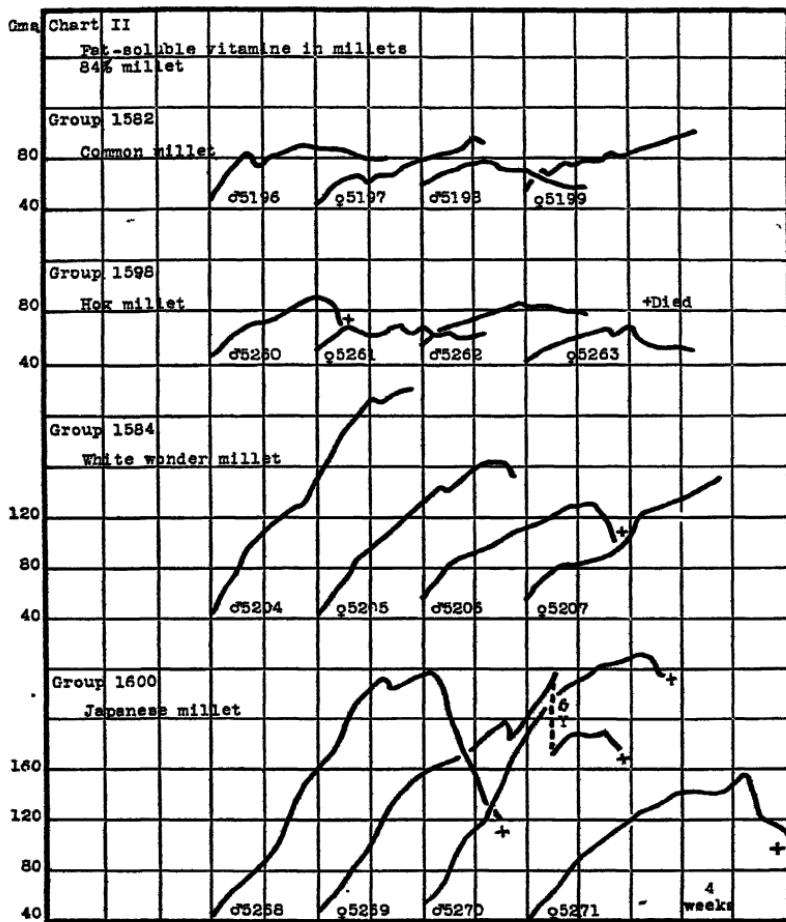


CHART II.

This theory would require that growth in the rats on both the Hog millet and Common millet was inhibited because of lack of the antirachitic vitamine because it represented the dominating deficiency. This appears very suggestive and if finally found tenable would mean that the so called fat-soluble vitamine content of grains and other foods must be studied with reference to the antirachitic vitamine as well as vitamine A.

The variations in fat-soluble vitamine content of millets suggested the possibility of harmonizing the data with the theory proposed by one of us (10) that the occurrence of the fat-soluble vitamine is intimately associated with certain yellow plant pigments. This, in fact, suggested that possibly the vitamine might be a yellow pigment or a related compound. This theory has aided us in our experimental work, but in the present state of knowledge cannot be absolutely disproven or proven as the knowl-

TABLE I.

	Japanese.	White Wonder.	Hog.	Common.
With chromate standard.....	100	190	140	150
" extract of Japanese millet as standard.....	100	180	120	130

edge of the chemistry of even the pigments themselves is too limited to allow profitable speculation. We have found a correlation between pigment and vitamine content in the case of white and yellow Indian corn (11), white and yellow carrots (12), white and yellow sweet potatoes (12), in different varieties of peas (13), and in cabbage leaves (12).

To determine if such relations exist in millets 15 gm. of finely ground millet were extracted for 6 hours with alcohol in a Soxhlet. The alcohol was evaporated off, almost to dryness, and the residue saponified over night with 20 cc. of 5 per cent alcoholic solution of potassium hydroxide freshly prepared with the usual precautions. The alcoholic solution was then diluted with water and shaken out repeatedly with ether until the ether extract came off colorless. The ether extracts were united, concentrated to 20 cc. volume, and compared one with another and with a standard composed of potassium chromate and potassium dichromate diluted to one-tenth the strength in which it was used formerly (14).

The comparisons were not entirely satisfactory as the colors were not of the same character—the extract from the Japanese millet especially having a pronounced greenish tinge, yet they represent fair approximations. Taking the pigment value of the Japanese millet as 100 the value of the others stand as shown in Table I.

They show that intensity of pigmentation cannot be correlated with vitamine content for while Common millet and Hog millet are low in both vitamine and pigment and White Wonder is somewhat richer in both, Japanese millet, the richest in vitamine, was the poorest of any in pigment. We have been unable to check up these observations as our supply of millet was exhausted, but it is possible that we have here in plant tissue a lack of correlation between pigment and vitamine as Palmer and Kempster have observed in pig's liver (15).

#### SUMMARY.

Millets are not uniformly rich in fat-soluble vitamine; some contain barely enough to give evidence of its presence, others may support growth at the normal rate in the rat for months, but failure ultimately supervenes.

It is questionable if it is warrantable to assume that millets occupy a unique position among grains with respect to their content of the fat-soluble vitamine.

It is also questionable if the relative size of germ to endosperm can be correlated with vitamine content, assuming that the proportion of vitamine is largest where the germ is the largest.

It appears that among the millets, fat-soluble vitamine content does not bear any definite relation to total yellow pigment.

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## FAT-SOLUBLE VITAMINE.

### XIII. LIGHT IN ITS RELATION TO OPHTHALMIA AND GROWTH.\*

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The therapeutic action of light has now been placed on a firm experimental basis by the work of Huldschinsky, Hess, Powers, and others.<sup>1</sup> These investigators studied the action of light, from various sources, in the prevention and cure of rickets and found that not only is it effective in promoting normal calcification of bone, but also in maintaining normal calcium and phosphorus content of the blood (2). Inasmuch as these same results can be secured by the addition of cod liver oil—a substance well known to be rich in the fat-soluble vitamine—the question immediately suggests itself if light can produce all the effects of cod liver oil, or more correctly, if light can produce all the effects produced by the fat-soluble vitamine. An accumulation of facts bearing on this matter cannot help but lead to a clarification of ideas in this field. Particularly profitable appears to be an analysis of the relations of light to ophthalmia and growth.

#### *Light and Ophthalmia.*

Sheets and Funk (3), and Powers, Park, and Simmonds (4) published data bearing on these relations at about the same time. The former took twelve rats and fed them a diet as low as possible in fat-soluble vitamine. Six animals were exposed to light either from a carbon arc or mercury-vapor lamp, while six, used as controls, were not. Three of each series came down with an ophthalmia at approximately the same time—the intervals varying from 128 to 140 days. This led them to conclude that light was without effect.

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<sup>1</sup> For a review of the literature on the effect of radiant energy on rickets see Park (1).

Powers, Park, and Simmonds carried out a number of experiments. In the first series they used fifteen animals of which they kept five under ordinary laboratory conditions of diffuse daylight, five in total darkness, and five exposed to radiation for a short period daily from a quartz mercury-vapor lamp. In a second series they used twenty-six animals. These also were divided up into three groups. All of the animals irrespective of treatment contracted ophthalmia; the time necessary for incidence of this affliction was, however, not stated. In the third series they used twelve rats treating three groups of three each as before, but including treatment with sunlight on the three remaining animals. A duplication of this experiment, making a fourth series, was run later with eighteen animals. The diet fed in these different series varied from one low in P, one low in good protein, to one low in protein of poor quality.

With respect to ophthalmia all the animals except those exposed to sunlight became afflicted early. In the sunlight group some developed it late, others had recurring attacks and two never developed it at all; in fact, all of them, with one exception, never developed ophthalmia until the animals in the other groups had already died from the disease. The authors conclude: "Sunlight probably exerts no specific antixerophthalmic influence but acts by raising the level of the cellular activity of the organism to the point where the progress of the disease is held in check or allowed to advance very slowly and with relatively little disturbance."

Hume (5) also carried out a number of similar experiments. In the first experiments she used six rats, two from each of three litters; one rat from each litter was used as a control and one was radiated 10 minutes every other day under a quartz mercury lamp. She emphasized the importance of using controls from the same litter because of "great difference in susceptibility to a vitamine A deficiency now known to exist between rats of different rearing." These precautions were omitted by the workers whose work was previously reviewed. This makes her observations of especial value because, as we have shown, the rat has tremendous capacity for storage of the fat-soluble vitamine which can account for a difference of months in the time of appearance of the ophthalmias (6). She found that the radiated animals "developed the typical symptoms of vitamine A deficiency with greater severity and greater rapidity than did the control animals" and this was particularly true of the ophthalmias. A fourth radiated animal, which had been given a different dosage of light than the aforementioned animals, also antedated its control in the incidence of ophthalmia by at least 14 days.

In a second series, Hume radiated eight rats which had already contracted ophthalmia and without fail observed an increase in the severity

of the symptoms. Her final conclusion was, that when radiated the ophthalmias become incident sooner and if present, before radiation is used, amelioration of symptoms does not result.

Goldblatt and Soames (7) duplicated Hume's results very closely, although their observations are not as extensive. They also speak of the necessity of taking storage of vitamine into consideration. All of five radiated animals developed ophthalmia and three out of five of the controls. On radiating rats after the incidence of ophthalmia their condition was aggravated.

Our results are rather fragmentary and bear in a limited way upon the problem under consideration, yet due to the large number of animals involved they are believed to be of value. We did not use intensified illumination, but attempted to determine if reduced light, that is darkness, had any effect on the incidence of the ophthalmias. As we have standardized our young rats with respect of age, size, and storage of fat-soluble vitamine for experimentation for this purpose we had every reason to expect to obtain results of value.

#### EXPERIMENTAL.

Forty-eight rats, twenty-four males and twenty-four females, were used for the trials. Six groups of four each were kept in dark cages and a corresponding number of cages exposed to the ordinary diffuse light of the laboratory. The environment with respect to feed, water, bedding, and runway was the same for all. The ration fed was composed of casein 18, salts 32, 4, agar 2, yeast 2, and dextrin 74. Both casein and dextrin had been thoroughly extracted with alcohol. The casein originally was a commercial product purified in the laboratory and the dextrin had been prepared from corn-starch. The yeast was part of a shipment which had been previously tested for its efficiency as a source of vitamine B.

The results obtained are presented in Table I. After very nominal growth, about one-fourth of what might be expected on a normal ration, ophthalmias became incident during the 5th week. There was no difference discernible in the rapidity of onset of the ophthalmias or their severity with degree of illumination. It was evident to us that variation in intensity of illumination within the range studied was not responsible for variations in

the occurrence of ophthalmias as we had observed them in our colony. As to whether illumination had any effect on the course of the disease we are unable to say as at this time we changed our animals to various vitamine preparations to test out their activity. The results of these studies will be published later. We feel justified in concluding that darkness as compared with diffuse daylight has no effect on the occurrence of the ophthalmias.

#### *Light and Growth.*

The investigators mentioned in connection with the studies of the influence of light on the incidence of ophthalmia also obtained results on growth in the same series of experiments.

TABLE I.

Groups.	No.	Albi-nos.	Hooded.	Average age when started. days	Weight when started. gm.	Increase in weight. gm.	Ophthal-mias at wks.
Light.	1534-1539	18	6	23.6 (23-26)	52 (42-67)	22.3 (12-35)	7 5-6
Dark.	1528-1533	12	12	24.5 (23-26)	58 (50-73)	18 (7-44)	7 5-6

Sheets and Funk (3) on two groups of rats started at the same age and approximately of the same weight, one radiated and one not, found that the radiated animals weighed from 72 to 110 gm. and the controls from 70 to 102 gm. at the close of the experiment. They conclude that there was no resultant difference in the rate of growth in the two groups whether radiated or kept under ordinary laboratory conditions.

Powers, Park, and Simmonds (4) unfortunately do not go into sufficient detail in their report on experiments with 71 animals to give the reader concrete data from which to draw conclusions with respect to growth.<sup>2</sup> They make a particular point of the effect of light on ophthalmia and rickets. Irrespective of light treatment they found that on a ration low in fat-soluble vitamine and phosphorus or good protein all the animals failed to grow.

<sup>2</sup> Since this was written these authors have published their work in detail in this Journal (Powers, G. F., Park, E. A., and Simmonds, N., *J. Biol. Chem.*, 1923, lv, 575).

Greater "bodily vigor" was promoted "to a very limited degree" by radiation with the quartz mercury-vapor lamp. "Rats exposed to sunlight made an initial gain in weight which in the animals on the rickets-xerophthalmia-producing diet was maintained and in those on the diet producing xerophthalmia alone never entirely lost." The reader is left with the impression that no marked influence on growth was observed.

Hume's (5) results were entirely different from those obtained by Powers and his coworkers. One of the reasons, though not the only one, as we shall point out later, was the selection of the experimental diet. The diet used by Hume was complete in composition except for the fat-soluble vitamine which it contained in very small amounts. It was not deficient in P nor good protein and therefore made growth possible when vitamine requirements or the equivalent were complied with. In her first series, the three radiated rats grew at a rate well above the normal of Donaldson for a period of 35 to 50 days and then flattened out. The three controls grew normally for 7 to 10 days then flattened out with subnormal growth. A fourth animal, radiated irregularly, grew normally till about the 50th day as compared with its control without radiation which grew normally for about 14 days. When the rats had been kept on the deficient diet for 91 to 158 days before radiation was started there resulted no resumption of growth with one exception where a temporary growth response resulting in an increment of 18 gm. was noted. When rats were kept for 17 days on the deficient diet without radiation prompt resumption of growth resulted upon exposure to the ultra-violet rays. This was maintained for 56 days. When the time of maintenance without radiation was increased to 35 days radiation again induced growth, but it was not so prolonged. These two trials can be accepted as giving the explanation why a resumption of growth had previously been observed in one rat. Undoubtedly these animals had not been kept long enough on the deficient diet before radiation was started.

The results of Hume are especially noteworthy on account of the precautions that she took to eliminate the storage factor which she considered proven by Korenchevsky's experiments (8). She demonstrated the effect of light when she used controls from the same litters and again when she eliminated the rôle of possible storage by making use of the recovery type of experiment.

Goldblatt and Soames used the same technique as Hume paying especial attention to using rats from the same litter as controls and determining maintenance of growth as well as recovery of growth by radiation with light from a quartz mercury-vapor lamp. In addition, however, they kept their animals, when not radiated, in a dark room. When they started light treatment from the very beginning the radiated animals gained weight more rapidly, the maximum weight attained was greater and growth ceased later than in the controls. This happened in two series including sixteen animals from 25 to 28 days of age. With the "recovery experiments" where radiation was started, respectively, after the rats had been 42, 49, and 56 days on the ration—all after longer intervals than those used by Hume—temporary resumption of growth occurred in the first two, but not in the last. In the main their findings agree with Hume's. The greater difference in growth between the radiated and control animals observed in Hume's experiments they attribute to the fact that the latter's animals were larger and older and probably had a greater store of vitamine A available.

In reading Hume's paper we became impressed with the remarkable response in growth resulting from radiation, but it appeared to us that this could not be due to its effect on vitamine A because in the control animals the incidence of ophthalmia did not become apparent until the 77th day. It seemed to us that if there was sufficient vitamine A present to delay the ophthalmia to such a late date there must have been enough present for normal growth for some weeks in the early periods of the experiment because we have seen ophthalmias occurring before growth or at least weight increments ceased, and in numerous instances have we seen ophthalmias making their appearance within 5 weeks after the cessation of growth. In fact, in experiments on the fat-soluble vitamine now running in our laboratory, we consider it very unusual if no ophthalmias appear in animals at this time. Furthermore, if light acts by economizing vitamine A or in liberating it more efficiently from stored reserves as suggested by Hume why should incidence of ophthalmia, a condition resulting from a fat-soluble vitamine deficiency, not be delayed. Light itself was not found harmful to the eyes and yet the radiated animals did not contract the ophthalmias later,

but even sooner and with greater severity than the controls. The hypothesis of Hume presents the paradoxical position that light liberates vitamine A for growth, but not for the prevention of the ophthalmias. It appears to us that another conclusion is far more justifiable and that is that growth in Hume's animals ceased because of an insufficiency of the antirachitic vitamine as distinguished from vitamine A. When its deficiency was compensated for by radiation, growth was restored and continued until vitamine A was exhausted; then ophthalmia made its appearance and rapid failure supervened. This theory leaves light without effect on vitamine A, it postulates that the antiophthalmic and antirachitic properties are resident in distinct entities as proposed by McCollum, Simmonds, Becker, and Shipley (9)—which is in direct opposition to Hume's conclusions—and it attempts to explain the variable results obtained by different investigators on the basis of variation in relative amounts of vitamine A and antirachitic vitamine in storage. We have submitted this theory to experimental inquiry and to date have accumulated the following data by the technique described.

In the selection of the proper conditions necessary for the demonstration of support for our explanation we realized that we were limited by the condition of our experimental rats with respect to their stored reserves of vitamine A and the proposed antirachitic vitamine. An inspection of numerous curves of growth obtained from young rats placed on a ration deficient in the so called fat-soluble vitamine, indicated that with few exceptions, previous to the onset of the inflamed eye condition, growth was at a standstill for a number of weeks. This is illustrated in Chart I where young rats from 22 to 24 days of age were put on a basal ration of alcohol-extracted casein 18, salts 32, 4, yeast 4, agar 2, and dextrin 72. These were selected at random from our records and do not tell the entire story because after 6 weeks on the above ration they were changed to various vitamine preparations. They suggested the possibility that, relative to their needs, these rats had stored away more vitamine A than antirachitic vitamine. If such was the case they ought all to respond to the addition of antirachitic vitamine or its equiva-

lent and then in spite of a response in growth, ultimate onset of ophthalmia ought not to be appreciably deferred and complete failure ought to supervene rapidly.

For our first series we selected sixteen young rats from 21 to 24 days old and weighing from 40 to 59 gm. They were divided into four groups in such a way that no two rats from the same litter were put in one group and as far as possible each litter was represented in each group.

The rats were kept on screens and fed a basal ration of casein 18, salts 40, 4, yeast 6, agar 2, and dextrin 70. The casein was a crude casein which had been heated in thin layers in a steam oven for 1 week and then thoroughly extracted with alcohol. Salts 40 was a synthetic salt mixture representing a reconstructed simplified salts 32 with KI addition. It had the following composition and has been used with much success in our laboratory.

	<i>mol.</i>	<i>gm.</i>
NaCl.....	4	233.6
MgSO <sub>4</sub> .7H <sub>2</sub> O.....	1	246
Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O.....	1	358
K <sub>2</sub> H PO <sub>4</sub> .....	4	696
Ca <sub>2</sub> H <sub>5</sub> (PO <sub>4</sub> ) <sub>2</sub> .4H <sub>2</sub> O.....	2	698
Ca(C <sub>2</sub> H <sub>5</sub> O <sub>2</sub> ) <sub>2</sub> .5H <sub>2</sub> O.....	0.5	154
Fe(C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> ) <sub>2</sub> .6H <sub>2</sub> O.....	0.1	59.8
KI.....	0.01	1.6

Two groups of rats were put on the aforementioned basal ration one of which, Group 2078, was kept continually under ordinary laboratory conditions in diffuse daylight and the other, Group 2081, was exposed 10 minutes daily, except Sunday, to the ultra-violet light of a quartz mercury-vapor lamp. A third group, No. 2080, was given 2 per cent aerated cod liver oil as prepared by McCollum, Simmonds, Becker, and Shipley (9), which represented a vitamine preparation containing the anti-rachitic vitamine but no vitamine A and a fourth group, No. 2082, was given the aerated cod liver oil and exposed to ultra-violet light as well.

In the second series of experiments we used the recovery type of experiment. Twelve young rats (six males and six females),

23 to 25 days old and weighing from 42 to 55 gm. were put upon the same basal ration as those of the previous series and allowed to fail in growth. When this had occurred which it had at the end of 23 days (in most of them it had occurred by the 19th day, except in the case of Rat 7328 which after being stationary for a week made a sudden gain in weight), one group, No. 2084, was exposed to ultra-violet light and kept on the basal ration; one group, No. 2079, had its ration changed to the extent that 2 per cent of aerated cod liver oil was added and the third, No. 2085, was given aerated cod liver oil and exposed to ultra-violet light as well.

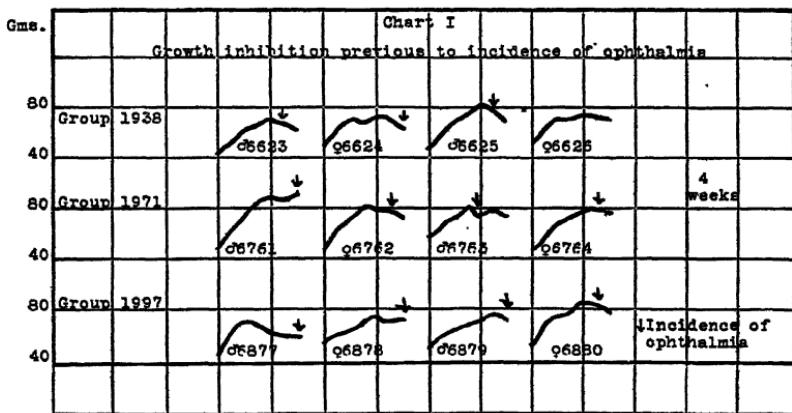


CHART I.

In both of these series we included the use of aerated cod liver oil because, if it is true, as claimed by McCollum, Simmonds, Becker, and Shipley (9), that when prepared under suitable conditions it contains the antirachitic vitamine and no vitamine A, feeding it should produce the same effect as light in these experiments. This necessitated first of all that we standardize the conditions necessary for destroying the antiophthalmic property of our oil.

McCollum, Simmonds, Becker, and Shipley (9) found that cod liver oil which had been aerated 12 to 20 hours at the temperature of boiling water was no longer able to cure ophthalmia in rats when administered to the extent of 2 per cent of the diet.

4 hours aeration was found insufficient. Unfortunately, these investigators do not present the data in detail in regard to the number of animals used in this standardization and as to whether or not the aerated oil was efficient at higher levels in curing ophthalmia. Viewed in the light of results reported by McCollum, Simmonds, and Becker (10) and Mori (11) that certain ophthalmias occur in the presence of a sufficiency of vitamine A—and presumably, therefore, do not yield to vitamine A therapy—and that these ophthalmias are grossly indistinguishable from each other such conclusions as were drawn by them are not at all convincing. At any rate as the vitamine content of cod liver oil varies and as aeration for so many hours at 100° is a process lacking in required definiteness, we restandardized the procedure both as to method of aeration and therapeutic effect for our supply.

The cod liver oil was aerated for varying periods of time; *viz.*, 30 minutes, 1, 2, 5, 10, 20, and 40 hours at 100°C. The oil was prepared in 400 cc. quantities contained in a bottle 9 cm. in diameter. The air was washed with alkali and acid, then filtered through cotton, and passed through a heated metallic coil to bring it up to the required temperature. It was drawn through the oil at the rate of 50 liters per hour. The temperature was maintained uniformly at 100° by putting both coil and container in a Freas oven set at that temperature. As the aeration proceeded the oil darkened in color. A slight change in its odor was also observed.

The therapeutic potency of the oil was established by incorporating 0.5, 2.0, 6.0, and 12.0 per cent of the treated oil in a basal ration of casein 18, agar 2, salts 32, 4, yeast 2, and dextrin 78; an amount of dextrin equal to the amount of oil added, being omitted as the modifications were made. The casein was a commercial casein, washed with water acidified with acetic acid (12) and then alcohol-extracted but not especially heated as in the previous experimental series.

108 rats in all, 42 to 69 gm. in weight and 3 to 4 weeks old, were used for the experiments. They were kept in groups of four on the basal ration for 42 to 46 days when invariably some of them showed signs of ophthalmias and the remainder was about to become so afflicted unless vitamine additions to the

ration were promptly made. When one trial did not give unequivocal evidence as to the antiophthalmic property of the preparation, a duplicate test was made and such repetitions were continued until all question of inactivity of the preparations was removed. The tests with 12 per cent of oil in the ration

TABLE II.

*Response of Ophthalmias in Rats to Different Amounts of Cod Liver Oil Which Had Been Aerated at 100° for Different Periods of Time.*

Time.	Amounts of cod liver oil.			
	0.5 gm.	2 gm.	6 gm.	12 gm.
30 min.	4 ophthalmias 4 cured.			
1 hr.	3 ophthalmias. 2 cured. 1 incident?	3 ophthalmias. 3 cured.	.	
2 hrs.	4 ophthalmias. 3 cured.	1 ophthalmia. 1 cured.	3 ophthalmias. 3 cured.	
5 "	4 ophthalmias. 1 cured.	6 ophthalmias. 2 cured.	8 ophthalmias. 6 cured.	.
10 "	1 ophthalmia. 0 cured. 3 incident.	10 ophthalmias. 2 cured. 2 incident.	8 ophthalmias. 1 cured.	7 ophthalmias. 3 cured.
20 "	2 ophthalmias. 0 cured. 2 incident.	3 ophthalmias. 0 cured. 1 incident.	3 ophthalmias. 0 cured. 1 incident.	4 ophthalmias. 0 cured.
40 "	2 ophthalmias. 0 cured. 2 incident.	1 ophthalmia. 0 cured. 3 incident.	2 ophthalmias. 0 cured. 2 incident.	3 ophthalmias. 0 cured.

were not entirely satisfactory as the ration containing it was not readily consumed by the animals.

The results obtained are presented in Table II. Ophthalmias were designated "cured" when cure was effected within 2 weeks after the addition, and "incident" when they showed up subsequent to the change of ration within the following 2 weeks. As the table shows, at the higher levels of intake 10 hours of aeration

at 100° was not sufficient to destroy all of the vitamine A. 20 and 40 hours of treatment on the other hand apparently destroyed all of it. For our experimental work we selected the material treated for 20 hours as we surmised that after 40 hours of aeration the antirachitic as well as the antiophthalmic property might have been destroyed, because we know from the experience of Hart, Steenbock, Hoppert, Bethke, and Humphrey (13) that the antirachitic factor is not a stable quantity.

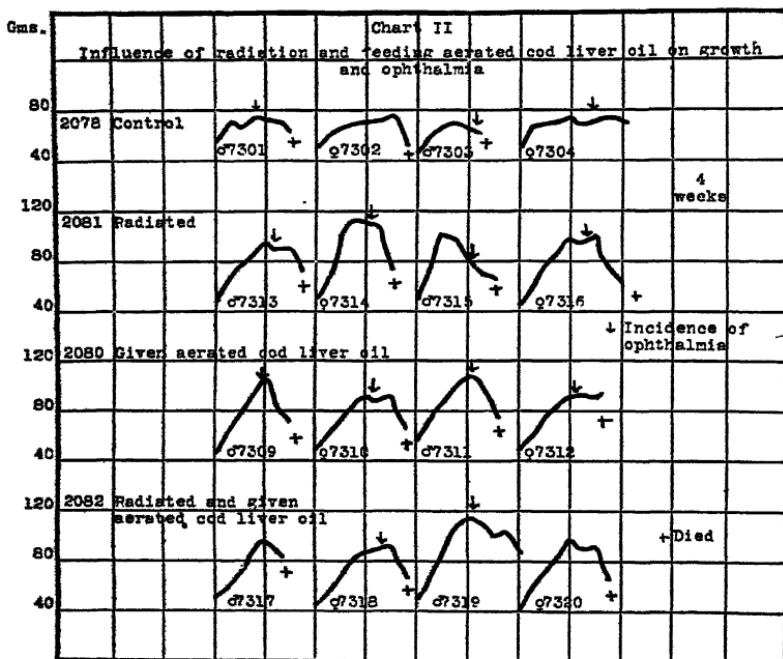


CHART II.

The results of the first series of experiments are presented in Chart II and Table III. Particular attention is called to the growth relations which show unequivocally that radiation or the feeding of aerated cod liver oil or both combined are efficacious and equally efficacious in promoting growth; without them growth practically ceases after a few weeks on the basal ration. The occurrence of ophthalmias and death was also very uniform but

this statement must be accepted with some reservation as one animal, Rat 7303, in the basal group was eviscerated by the others. Although we have shown that the viscera become impoverished in vitamines (6) with vitamine starvation the possibility that the occurrence of death and ophthalmias was postponed by vitamines thus obtained is not excluded.

TABLE III.

*Detailed History of Animals the Curves of Growth of Which Are Shown in Chart II.*

Change of ration or treatment.	No.	Sex.	From litter No.	Age. days	Initial weight. gm.	Maximum weight. gm.	Incidence of ophthalmia. days	Death. days
2078 Basal.	7301	♂	2	25	54	73	23	41
	7302	♀	5	24	48	77?	R	52
	7303	♂	1	25	45	67	30	32
	7304	♀	4	25	52	75	39	
2081 Radiated.	7313	♂	4	25	47	94	34	47
	7314	♀	1	25	48	114	32	47
	7315	♂	8	21	50	115?	30	47
	7316	♀	3	25	45	102	39	55
2080 Given aerated cod liver oil.	7309	♂	3	25	44	110	27	42
	7310	♀	7	24	50	91	30	50
	7311	♂	2	25	59	113	32	42
	7312	♀	1	25	47	95	32	46
2082 Radiated and given aerated cod liver oil.	7317	♂	4	25	52	97	R	37
	7318	♀	1	25	45	92	39	
	7319	♂	6	23	50	118	32	53
	7320	♀	3	25	40	98	R	46

R denotes respiratory infections.

We believe that these experiments support our theory. In the basal group, growth was maintained for the first few weeks until the reserves of antirachitic vitamine were depleted. Then growth ceased, but maintenance was possible as long as the vitamine A stores were not exhausted. With their exhaustion, ophthalmia or infections of the respiratory tract occurred and death followed. When, on the other hand, the antirachitic factor was supplied from the beginning, either as vitamine or light,

cessation of growth, when it did occur, occurred because of depletion of the stored reserves of vitamine A and ophthalmias and death resulted as before in spite of greater growth.

The data of the second series of experiments are shown in Chart III and Table IV. They show in the first place a very decided response in growth with exposure to ultra-violet light or the addition of aerated cod liver oil. The responsiveness of the animals was indeed quite remarkable. With one exception and that in the case of Rat 7332 which lost weight for 3 days before

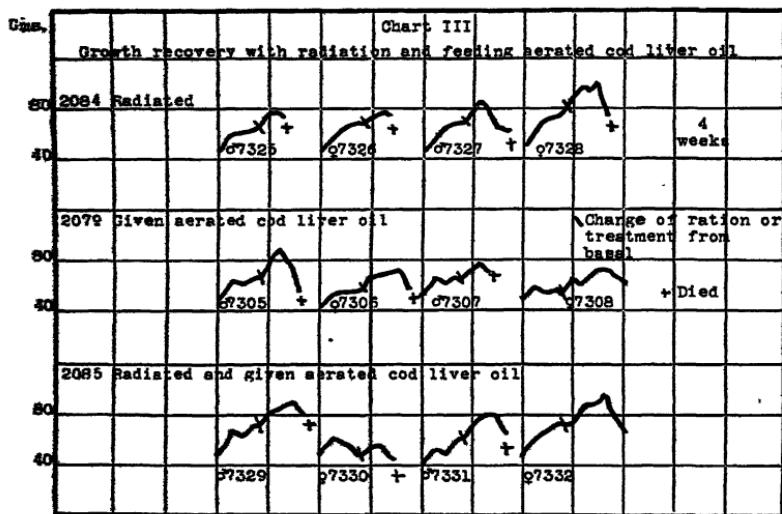


CHART III.

responding, all the animals responded immediately. Yet in spite of this the improvement was not permanent. With the exhaustion of the reserves of vitamine A ophthalmias became incident and death resulted. Here also our conclusions are limited by the early death and consumption of the viscera of Rat 7326 by the other rats of the group. As this occurred on the 36th day of the experiment it is possible that Rats 7327 and 7328 had the onset of their ophthalmias delayed as a result of vitamine A thus secured. However that may be, it appears certain from the behavior of the other animals that aerated cod liver oil was free from the antiophthalmic factor and that such

effects as were produced by it were analogous to those produced by light.

We were able to confirm the experience of Hume (5) and Goldblatt and Soames (7), that radiation is without effect when applied later in the period of maintenance. We tested this out on two animals which had been on the purified ration for 41 days. Both had been practically stationary in weight for 30 days and

TABLE IV.  
*Detailed History of Animals the Curves of Growth of Which Are Shown in Chart III.*

Change of ration or treatment.	No.	Sex.	From litter No.	Age.	Initial weight.	Change in weight.		Incidence of ophthalmia after starting experiment.	Death.
						4 day average previous to change.	Day after change.		
2084 Radiation.	7325	♂	4	25	45	+0.5	+3.0	32	39
	7326	♀	1	25	45	+0.25	+2.0	27	36
	7327	♂	6	23	45	+0.25	+3.0	48R	54
	7328	♀	5	24	50	+2.00	+5.0	39	46
2079 Aerated cod liver oil.	7305	♂	7	24	50	+0.25	+2.6	30	47
	7306	♀	2	25	44	+0.00	+3.6*	30	51
	7307	♂	1	25	55	-0.50	+2.6	30	45
	7308	♀	1	25	55	-0.50	+4.0*	32	
2085 Radiation and aerated cod liver oil.	7329	♂	5	24	52	+0.00	+5.0	32	49
	7330	♀	2	25	50	-0.50	+0.5	32	46
	7331	♂	6	23	42	+0.50	+1.0	30	46
	7332	♀	5	24	48	+0.00	-1.0	27	

\* These animals were not weighed till the 3rd day after the change, therefore a daily average for this period is given instead.

R denotes respiratory infections.

both had ophthalmias. One died 5 days after the radiation was begun, while the other did not die, but neither did it begin to grow in the 5 days that it was kept under observation. This result we believe bears out our assumption that the animal is able to respond to the antirachitic agents only as long as vitamine A is present in amounts sufficient for the animal's needs. When this fails, death results.

We believe that all difference of opinion can be reconciled by taking into consideration the possibility of vitamine storage and assuming that in the growth phenomena on a ration free from vitamines except those carried by yeast we have to deal with at least two vitamines; namely, vitamine A and the antirachitic vitamine. Hume (5) assumed that because light had the same effect on growth as she expected to get with what has been known as vitamine A that the probability of identity of vitamine A and the antirachitic vitamine was heightened. We believe that her results show exactly the opposite as such an assumption leaves unexplained the fact that the ophthalmias were neither prevented nor deferred. Her rats were able to grow exposed to ultra-violet light only as long as the stored reserves of vitamine A made growth possible. Without the influence of light, growth was inhibited sooner, because, in proportion to the animal's requirements, less of the antirachitic than of the antiophthalmia factor had been stored away. When finally the reserves of vitamine A were exhausted rapid failure resulted in both cases.

Powers, Park, and Simmonds (4) did not pay a great deal of attention to growth in their experiments. Lack of standardization of their animals and the possibility that their animals had equal or nearly equal amounts of the two vitamines on reserve made demonstration of a difference difficult. They do, however, state that ultra-violet light "promotes bodily vigor to a very limited degree." In contrast to Hume they conclude that "By analogy, therefore these experiments suggest that there are in cod liver oil two distinct factors one preventive and curative of rickets and the other preventive and curative of xerophthalmia. Sunlight can compensate for the absence of one but not completely of the other."

When we associate—as we do in this paper—failure of growth under certain conditions with lack of antirachitic vitamine we realize that our theory runs counter to the conclusions of many that rickets is a phenomenon for the production of which growth is necessary. Park (1) in his excellent article on rickets dismisses the matter by saying,<sup>3</sup> "For the development of manifest

<sup>3</sup> Park (1), p. 137.

rickets and osteomalacia growth is essential. Both are diseases intimately concerned with the phenomena of growth. . . . So many writers have pointed out the fact that malnourished infants do not become rachitic that one need only mention the fact here."

We hold no special brief for arguing that we are dealing with rickets as we realize fully that our evidence, based merely on restoration of growth by light and aerated cod liver oil, is of a very indirect nature and that we are in no position to pass judgment on clinical observations. We are even willing to admit that in many cases growth is absolutely necessary to produce rickets as thereby the animal exhausts its reserves of the antirachitic factor. Without a sufficiency of other growth-promoting agents, pathological conditions other than rickets gain the ascendancy, the reserves of the antirachitic factor are not exhausted and rickets does not make its appearance.<sup>4</sup> Such a theory for instance would explain why a certain amount of vitamine A in the diet is necessary for the production of severe rickets in rats.<sup>5</sup> Without it in the diet, unless the animal has a large reserve of it stored away, the animal fails completely from ophthalmia and respiratory infections before the reserves of the antirachitic factor are exhausted and rickets can become manifest.

We believe it highly probable, however, that in its etiology with respect to vitamine relations rickets is not such a distinct entity as has been assumed. It undoubtedly is very closely related to the failure of calcium retention as observed in the goat by Steenbock, Hart, and Hoppert (14), the main difference being that in the growing animal in the absence of the antirachitic factor a unique anatomical picture in bone structure makes its appearance. It is highly probable that the presence of the vitamine or its equivalent may be a requirement for normal calcium metabolism of all cells, but as osseous tissues are predominantly concerned with the metabolism of this element in rather unique relations its importance has there been emphasized. If such should be the case, we may not be so far from the truth in assuming that growth was inhibited in our animals by a lack of the antirachitic vitamine.

<sup>4</sup> It is, of course, admitted that there are other factors besides the antirachitic vitamine which modify the assimilability of the element Ca.

<sup>5</sup> Park (1), p. 131.

The conclusions and opinions voiced in this paper are to be considered as preliminary to further consideration. We believe them worthy of confirmation or condemnation as the case may warrant in the furtherance of knowledge in this field. We wish to hold no reservations as to future work.

#### CONCLUSIONS.

Young rats raised on our stock ration and transferred to a purified ration carrying yeast as its only source of vitamines will grow for a few weeks then cease growing completely or partially and ultimately will fail due to the incidence of ophthalmia or infections of the respiratory tract. Aerated cod liver oil or light from a quartz mercury-vapor lamp, both well known as antirachitic agents will eliminate the initial failure of growth, or, when prevalent, will restore it without appreciably postponing the final failure due to ophthalmia or respiratory diseases.

Inasmuch as our aerated cod liver oil did not cure ophthalmia the experiments lend support to the theory of McCollum that cod liver oil contains two vitamines; *viz.*, vitamine A and the antirachitic vitamine.

It is emphasized that in the study of growth or incidence of pathological conditions as provoked by the feeding of deficient diets it is necessary that due attention should be paid to the vitamine reserves of the animal. With this under control the possibility presents itself of using growth in the rat for the demonstration of the presence of a fat-soluble vitamine other than vitamine A, presumably the antirachitic vitamine, in our foods and feeding materials. Furthermore, with the antirachitic factor supplied vitamine A can now be determined.

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## THE AVAILABILITY OF CALCIUM SALTS.\*

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One of the hypotheses that has been presented in an attempt to explain deficient calcium assimilation during periods of abundant calcium intake is that the calcium is presented in a form unavailable to the animal body. Curiously enough it appears that this hypothesis has never been subjected to rigorous experimental treatment. In the last few years this has, however, been brought to the fore, for with the understanding that recognition of a vitamine influencing calcium metabolism has given us, experiments solving these relations can be successfully performed.

In experiments growing out of the realization that grains are woefully deficient in calcium, in relation to the requirements of pigs for economical pork production, it was shown more than a decade ago, at this Station, that calcium can be successfully added in the form of phosphate (1). Aron and Frese (2) as early as 1908 combatted the all too prevalent idea that mineral elements must be in organic combination to make normal assimilation possible. As a result of experiments with dogs they concluded that the growing dog can obtain its calcium just as well from tertiary calcium phosphate as from milk; in fact, the availability of inorganic calcium seemed plausible to them as ordinarily the dog obtains its lime from bones in which it is present as a phosphate carbonate complex.

We had our attention drawn to this subject in 1912 when we noticed a very pronounced negative calcium balance in a goat fed on grains and straw (3). Testing out the solubility of the calcium in the straw we found it all soluble in 0.05 N HCl which made it

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appear questionable that it was present in an unavailable form. We were unable to explain the actual loss of lime from the body of the animal until we observed that on this same ration a positive balance could be induced by feeding the animal temporarily on oats and pasture. These observations correlated with the well known and recently intensively studied relation of cod liver oil to rickets led one of us<sup>1</sup> to suggest the presence of some substance in green plant tissue functioning in the assimilation of calcium. This has been supported by later studies from this laboratory (4). These studies emphasize emphatically the necessity for the accumulation of data on the various factors which operate in the assimilation of calcium not only with respect to assimilation from the gut but from the body fluids by the various tissues concerned.

One point of view which has been current in the literature, as the result of gross clinical observations, is that calcium soaps are unassimilable. Just why this assumption should have been made is not clear, but apparently it had its origin in the observation of Herter (5) that in infants growing slowly and rachitic an excessive amount of calcium is often excreted as the calcium soap. Grulee (6) states: "It is known that  $\text{Ca}_3\text{PO}_4$  is almost insoluble and probably not absorbed to a great degree and it is very likely that calcium is not absorbed in the form of calcium soaps." Hill (7) states: "The fat absorption becomes poor as so much of the fat is changed into insoluble calcium soaps which are absorbed with relative difficulty." It is but fair to emphasize the fact that both of these authorities ultimately leave the impression with the reader that the formation of the calcium soaps and resultant failure of absorption may be a secondary matter in connection with other disturbances.

McClugage and Mendel (8) bring up another matter. They show in experiments with dogs that calcium is not assimilated as well from carrots and spinach as from milk or the carbonate. They call attention to Bertram's statement (9) that the absorption of calcium from vegetable foods is poorer than from animal foods. They state that a number of factors are involved, but specifically emphasize the rôle of an increase in the excretion of indigestible material which may hinder the reabsorption of lime

<sup>1</sup> H. Steenbock.

secreted into the intestine. Voit (10) in 1880 also called attention to the importance of foods in causing negative calcium balance through excessive production of feces. Rose (11) and Blatherwick and Long (12), in experiments on adult man, show that calcium can be assimilated successfully from carrots, but Sherman and Hawley (13), in children 3 to 13 years old, show that while vegetables may serve as a source of calcium they are inferior in value to milk when compared on the basis of equal calcium intake.

There are, no doubt, other factors operative in calcium retention such as the form of salt combination and the dissociation of the calcium compounds. Givens and Mendel (14) report: "The striking fact is, that 0.34 gms. of calcium oxide per day in 21.7 gms. of dried skimmed milk, regardless of base or acid, will produce a positive calcium balance whereas 1 gm. of calcium oxide in the form of calcium lactate is necessary to accomplish the same end. The problem of the relative advantage of the combination in which calcium is fed is thus raised anew." For some reason or other calcium lactate does not seem to be an especially good source of calcium for the animal. 20 gm. daily for man have been reported to call forth untoward symptoms (15). We have also observed in some experiments on growth in rats that calcium lactate does not constitute an especially good source of calcium for the animal.<sup>2</sup> However that may be, the time appears opportune to investigate the availability of various calcium salts where excessive production of feces is avoided and where the ration is abundantly supplied with the vitamine which influences calcium retention. Our experiments on this have been outlined from 1920 to date.

#### EXPERIMENTAL.

All our experiments so far have been performed on the rat, using growth as the criterion of the availability of the calcium supplied. The rats were fed, four in a group, each group in a cage 2 feet square and bedded with shavings. They were given distilled water, fed daily, and were weighed once a week.

In the first series, which was considered in the nature of a preliminary trial, there was fed a basal ration of yellow Indian

<sup>2</sup> Unpublished data.

corn 50, purified casein (16) 13, butter fat 5, dextrin 32, sodium chloride 1, and iron citrate 0.14. To this there were added various calcium salts as it was already known to us from other work that the ration carried a sufficiency of other elements to allow normal growth.

The calcium was added to the extent of approximately 0.3 of a per cent—actually 0.295 to 0.298 per cent—respectively, in the form of calcium carbonate, 0.74 gm.; calcium lactate, 2.22 gm.; calcium sulfate, 1.27 gm.; tricalcium phosphate, 0.76 gm.; and calcium silicate, 2.24 gm. The carbonate was a Merck's U.S.P. IX preparation, the lactate, Mallinckrodt's, and the sulfate, Baker's analyzed. The phosphate and silicate were prepared in the laboratory.<sup>3</sup>

For the phosphate 196 gm. of  $H_3PO_4$  in 23 per cent solution were evaporated on 300 gm. of  $CaCO_3$  to dryness and then heated to redness in an electric muffle. There were obtained 310 gm. of tricalcium phosphate, analyzing 39.9 per cent calcium. Theoretically it should have contained 39.6 per cent calcium.

The silicate was prepared by precipitation of 219 gm. of calcium chloride with 215.3 cc. of water glass solution of 20 per cent  $SiO_2$  content. The precipitate was washed free from chlorides and dried at 110° giving 192 gm. of a product which analyzed 30.8 per cent silicon and 13.3 per cent calcium. This evidently represented a mixture of calcium silicates,  $2CaO \cdot 9SiO_2 \cdot 3H_2O$  theoretically containing 35.6 per cent silicon and 11.3 per cent calcium, and  $CaSiO_3$  containing 30.8 and 13.3 per cent of these elements.

The results obtained in the feeding trials using these materials are shown in Table I. Growth in all the groups receiving the addition of calcium salts is practically the same and all show marked superiority to the growth of the basal group. The animals in the basal group showed abnormalities in locomotion, walking with a peculiar stiff legged, crouching, shambling gait, and some individuals showed a deformity of the vertebral column in the lumbar region which undoubtedly contributed as much to the peculiar walk as weakness in the limbs themselves. As the experiment progressed, the basal animals, with one exception,

<sup>3</sup> Prepared by Dr. P. W. Boutwell, formerly of this laboratory.

Ration.	No.	Age months	Sex. weight.	2nd	4th	6th	8th	10th	12th	13th	14th	Remarks.
			gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
Basal.	2352	♂	70	88	93	90	90	80	85	82	77	Rat 2355 did not develop an ophthalmia. All the others did; Rat 2352 at 7 weeks; Rats 2353 and 2354 at 10 weeks.
	2353	♂	52	75	87	90	85	80	+			
	2354	♂	62	78	94	94	114	115	100	109	102	
	2355	♀	58	75	83	89	95	97	85	70	+	
Basal + calcium lactate.	2360	♂	66	103	132	163	190	200	207	219	237	
	2361	♀	59	94	117	143	157	183	187	215	242	
	2362	♂	49	77	103	129	170	200	215	235	260	
	2363	♀	50	69	87	107	124	140	157	147	157	
Basal + calcium carbonate.	2366	♂	79	116	150	185	201	213	220	240	240	Rat 2367 had young the 10th week, but did not raise them.
	2367	♀	88	107	135	173	164	157	140	160	175	
	2368	♂	58	105	140	175	203	213	218	235	265	
	2369	♀	38	55	75	100	108	113	120	127	135	
Basal + tricalcium phosphate.	2372	♂	74	95	130	152	164	175	178	185	190	
	2373	♀	75	96	126	147	152	166	172	204	186	
	2374	♂	62	70	95	130	149	161	180	190	244	
	2375	♀	47	78	89	112	125	128	122	125	130	
Basal + calcium sulfate.	2364	♂	54	83	99	123	150	184	202	215	218	Rat 2367 had young the 14th week, but did not raise them.
	2365	♀	45	75	101	116	130	149	165	160	174	
	2366	♂	48	71	95	113	132	153	165	185	192	
	2367	♀	76	102	128	139	150	168	170	225	180	
Basal + calcium silicate.	2368	♂	76	124	157	180	193	210	218	230	252	
	2369	♀	62	120	138	127	169	177	180	180	190	

developed an ophthalmia and one rat showed signs of a rhinitis. These last mentioned symptoms of an infectious nature were not observed in the groups where calcium was added; nevertheless, we believe that their occurrence probably was not related to absence of calcium salts directly. As a result of the low calcium and vitamine intake on the basal ration, growth was soon inhibited and decreased consumption resulted. As all our rations were made up in kilo quantities, the basal ration naturally was not made up as frequently as the others. This allowed sufficient lapse of time for the antiophthalmic factor added with the cod liver oil to be destroyed by atmospheric oxidation in this group in contrast to the others.

In Figs. 1, 2, and 3 are shown photographs of three male rats taken at the 15th week of experimentation, respectively, from the basal, the calcium sulfate, and the calcium silicate groups. The photographs bring out not only the difference in size but also show the ophthalmia, rhinitis, and spinal deformity characteristic of the basal animals.

The second series was started in duplication of the first, using purified and analyzed food constituents so that the calcium intake was controlled. As a basal ration there was used a ration of casein 18, agar 2, cod liver oil 2, yeast 2, dextrin 73.6, and salt 39, 2.4. The casein was a crude commercial casein which was washed with 0.2 per cent HCl as long as possible until it went into the gel state. It was then precipitated as a curd with dilute sodium hydroxide and washed repeatedly with distilled water. The final calcium content was reduced to 0.019 per cent. The agar, yeast, dextrin, and cod liver oil were drawn from our usual stock.

Salt 39 was a calcium-free salt mixture made up in the same proportions as our salt 32 (17) except for the calcium lactate and phosphate which were left out. In 2.41 gm. of this dried salt there was obtained the same intake of the inorganic elements as with 4.1 gm. of salt 32 with the exception of the calcium—which it was desired to reduce—and of phosphorus. The phosphorus was reduced from 0.6 to 0.329 gm. This still left a large excess over the requirements of the animal.

Into this basal ration there was introduced, at the expense of dextrin, calcium to the extent of approximately 0.4 per cent

in the form of calcium salts, the same as in the previous series with the addition of a Merck's calcium silicate, labelled M by us for comparison with our own preparation labelled B.

The actual amounts of salts fed per 100 gm. of ration and their Ca equivalents were as follows:

Salt.	Fed. gm.	Calcium equivalent. gm.
Calcium lactate.....	3.1	0.415
" carbonate.....	1.0	0.400
" phosphate.....	1.0	0.389
" sulfate.....	1.7	0.398
" silicate B.....	3.0	0.399
"      " M.....	3.0	0.436

In addition, there was fed to one group of animals the basal ration from which salt 39 was left out and 4.1 gm. of salt 32 were added. This carried a calcium content of 0.400 gm.

The constituents of the basal ration together gave a total of 0.039 gm. of Ca per 100 gm. With the salt additions, therefore, the calcium intake varied from 0.428 to 0.475 which provided a great sufficiency of calcium if available.

The results of this trial are shown in Table II. The data bring out conclusively that when calcium is fed in liberal quantities to the rat, the latter can cover its requirements with any of the well known calcium salts. So far as indicated there was no difference in their relative availability. This latter question of comparative availability is, however, still an open question because it is apparent that even the slightest solubility of the most insoluble calcium salt used might still allow the solution of enough calcium if the irrigation of the intestinal contents with digestive secretions is sufficiently large. If in addition, the animal's needs are not much larger than the content of the basal ration, a slight solubility would mean sufficient assimilation. We have reasons to believe that the total amount of calcium required for normal growth is not near as large as the calcium administered with these various salts.

In the third series of experiments we reduced the calcium intake to one-fourth the amount fed in the preceding series. The basal

## Availability of Calcium Salts

TABLE II.

Ration.	Rst No.	Sex.	Initial weight.	Weeks.							
				2nd	4th	6th	8th	10th	12th	14th	16th
				gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Basal.	5802	♂	69	94	105	112	110	113	117	133	132
	5803	♀	59	77	80	81	75	80	79	95	97
	5804	♂	60	88	98	103	106	104	93	100	95
	5805	♀	63	91	97	98	91	90	90	110	104
Salt 32.	5806	♂	59	89	128	165	200	237	252	268	270
	5807	♀	59	92	125	144	166	192	200	200	205
	5808	♂	60	99	155	189	232	275	297	318	325
	5809	♀	57	92	115	135	172	194	216	242	235
Calcium lactate.	5830	♂	60	105	142	172	194	226	244	260	268
	5831	♀	54	78	112	137	145	163	180	194	185
	5832	♂	50	88	118	150	170	203	217	240	230
	5833	♀	59	85	107	120	133	153	163	170	150
Calcium carbonate.	5822	♂	55	96	138	181	216	252	261	268	278
	5823	♀	57	91	117	140	156	174	190	196	212
	5824	♂	60	96	121	175	210	242	276	305	317
	5825	♀	56	90	120	137	162	192	212	208	215
Tricalcium phosphate.	5826	♂	60	106	145	194	244	272	282	310	323
	5827	♀	55	84	110	130	165	185	202	202	200
	5828	♂	50	83	127	163	208	259	265	275	290
	5829	♀	60	73	104	122	145	177	162	182	185
Calcium sulfate.	5810	♂	62	95	135	164	191	+			
	5811	♀	64	95	132	161	185	216	217	222	229
	5812	♂	59	94	130	165	192	222	232	257	257
	5813	♀	58	88	117	137	170	185	195	202	205
Calcium silicate B.	5814	♂	65	107	160	207	247	287	300	339	343
	5815	♀	62	95	131	156	178	215	197	233	230
	5816	♂	54	99	162	191	227	250	254	272	280
	5817	♀	60	87	113	126	141	162	150	169	178
Calcium silicate M.	5818	♂	60	90	137	192	226	268	288	298	292
	5819	♀	60	94	124	138	165	200	207	216	215
	5820	♂	64	105	167	195	227	272	285	293	303
	5821	♀	57	85	117	135	152	192	195	201	197

ration remained the same and the salts fed were the same with one exception and that was the tricalcium phosphate. This was a Mallinckrodt preparation carrying only 33.81 per cent Ca by actual analysis instead of the 39.6 required by theory. Apparently it was a mixture of the dicalcium and tricalcium salts.

The actual amounts of these salts fed and the calcium equivalents were as follows:

Salt.	Fed. gm.	Calcium equivalent. gm.
Calcium lactate.....	0.8	0.107
"    carbonate.....	0.25	0.100
"    phosphate.....	0.3	0.101
"    sulfate.....	0.4	0.094
"    silicate M.....	0.7	0.102

With the calcium of the basal ration they bring the calcium intake per 100 gm. of ration up to 0.133 to 0.140 gm.

The results of this trial are shown in Chart I. They are consistent in revealing no differences in the availability of the calcium salts. We must admit, however, that we are not entirely convinced that slight differences may not exist. We intend to repeat this work, using still lower levels of calcium intake with the added precaution of keeping our animals on screens to prevent consumption of bedding as well as feces (16). We do, however, believe that we are entirely justified in concluding that all of these salts are available to a considerable degree as a source of calcium for growth in the animal.

In the earlier literature it is easy to understand why the idea gained credence that calcium salts insoluble in water or dilute alkali were unavailable because the content of the small intestine shortly distant from the pylorus was believed to be alkaline in reaction. It is now known, however, that the acidity of the chyme is neutralized gradually and an acid reaction often prevails for a considerable length of the small intestine and even sometimes throughout its entire length (18).

It is undoubtedly the acidity which makes possible the assimilation of the difficultly soluble calcium salts from the intestine. Salts such as barium sulfate (insoluble in dilute hydrochloric acid)

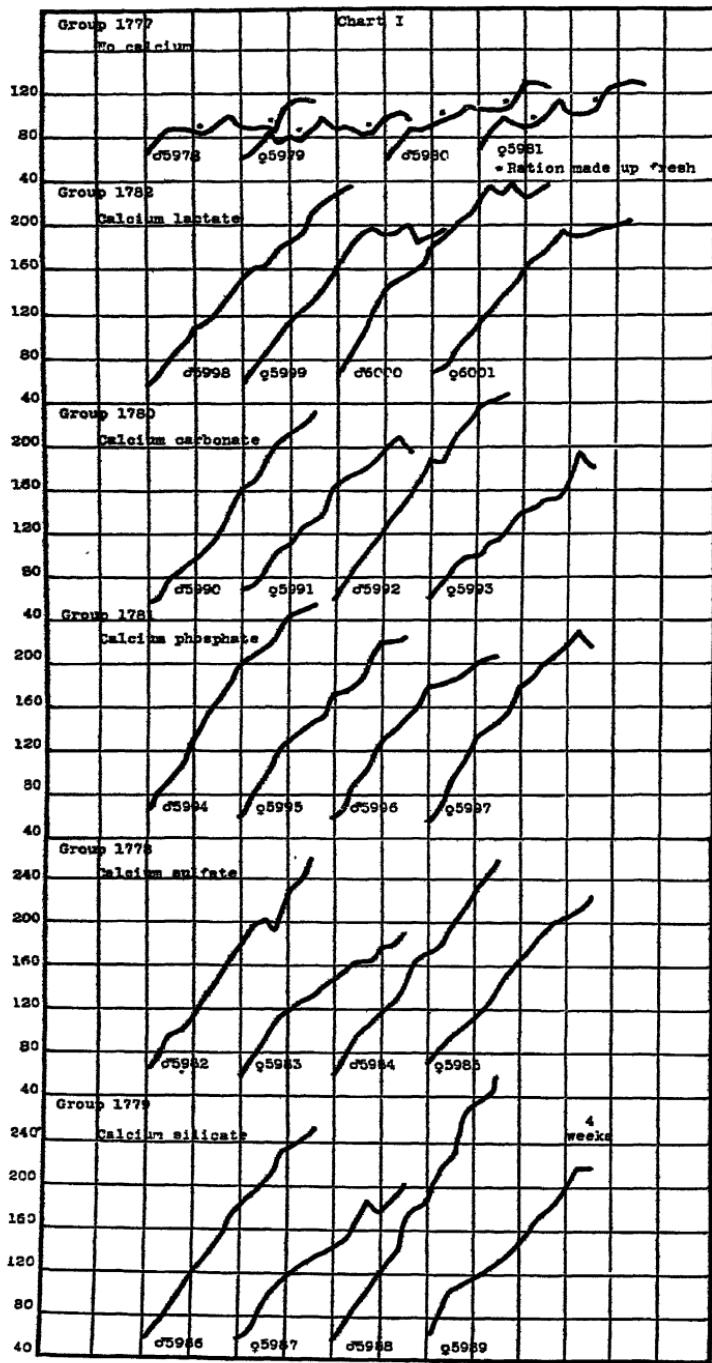


CHART I.

are not absorbed to an appreciable degree, at least not sufficiently to cause toxic symptoms. We determined this by feeding barium sulfate to two groups of rats, one on the basal ration used for the calcium work with salt 39, and one with the same plus salt 32. In each case 2 per cent of BaSO<sub>4</sub> was added. With salt 39, the incomplete salt mixture—which due to lack of calcium is unable to support growth—four rats started at 42 to 52 gm. in weight at an age of 24 days maintained themselves with slight growth for 18 weeks. At this time they weighed 77 to 93 gm. with no signs of immediate failure. All of them were, however, extremely rickety as indicated by their walk due to the calcium deficiency. With the complete salt mixture, salt 32, in the basal ration, plus the barium sulfate, the rats started at 42 to 52 gm. at an age of 25 days grew normally, attaining weights of 178 to 275 gm. in 18 weeks with no signs of failure.

We desire to call attention to the irregularity of growth on the basal ration which we were at a loss to understand until we looked up our data on consumption. We then found that each time that the animals gained weight the ration had been made up fresh. Apparently the cod liver oil of the ration had undergone deterioration in vitamine content with aging. Of the deterioration there can be no question. It did not become apparent in the groups where calcium was added, because the 1 kilo lots, in which the rations were made up, were consumed sooner. We are inclined to believe that the data show that with larger calcium intake less vitamine is required for normal growth. These relations will be discussed in a later publication.

#### CONCLUSIONS.

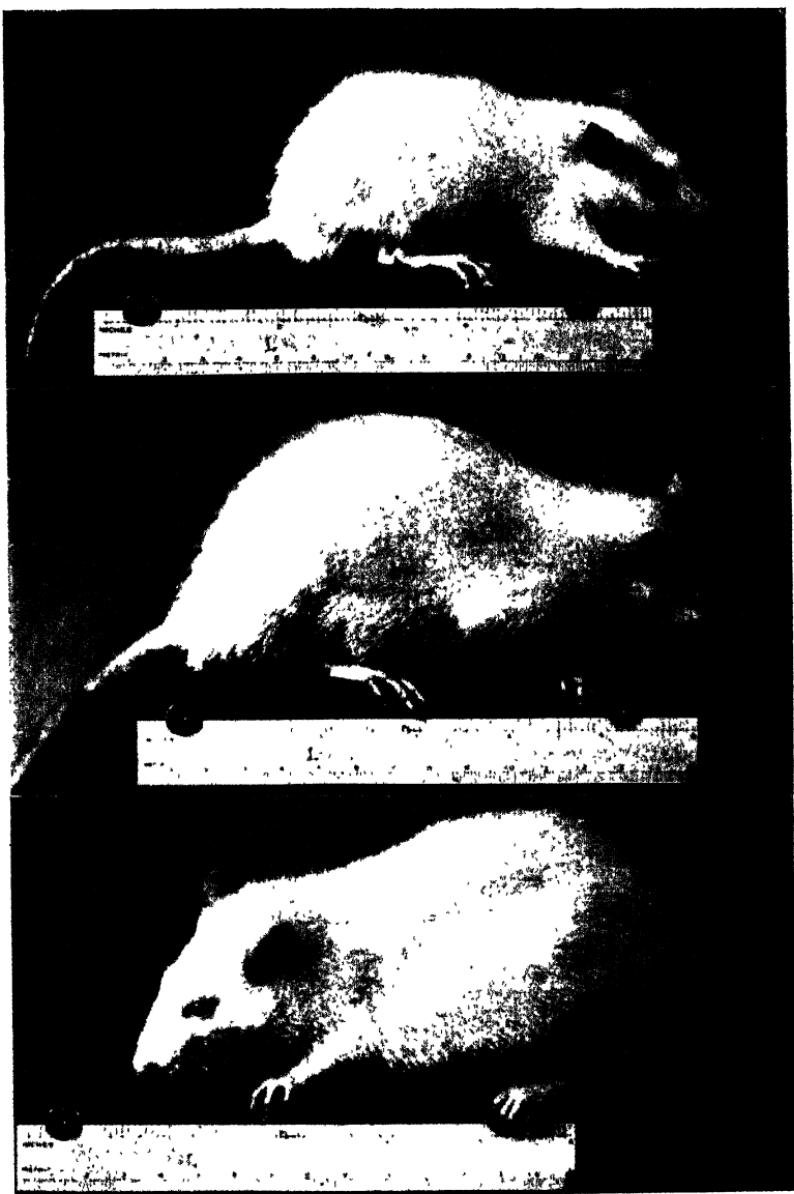
In experiments with young rats no difference was found in the availability of calcium lactate, carbonate, phosphate, silicate, or sulfate when these were fed in liberal amounts.

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## EXPLANATION OF PLATE 1.

Three representative male rats, Nos. 2352, 2364, and 2368, taken respectively, from Group 588, the basal group, Group 591, the calcium sulfate group, and Group 592, the calcium silicate group, photographed 15 weeks after the beginning of the experiment. Initially they weighed, respectively, 70, 54, and 75 gm. When photographed they weighed 82, 219, and 246 gm. With the inhibition of growth on the basal ration is to be noted the ophthalmia, the rhinitis, and the peculiar posture.



(Steenbock, Hart, Sell, and Jones: Availability of calcium salts.)



## FRUCTOSE, GLUCOSE, AND GALACTOSE TOLERANCE IN DOGS.

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In a recent study, Folin and Berglund (1) describe certain phenomena associated with the transportation, retention, and excretion of carbohydrates. Data are presented to show that both fructose and galactose are less effective than glucose in raising the level of the blood sugar. The explanation offered is that the tissues, being relatively well stored with glucose and empty of other sugars may be able to absorb these other sugars more readily than glucose. Quite different results have been reported by Foster (2, 3). The latter finds that the ingestion of 40 to 100 gm. of galactose by normal adults produces a very marked hyperglycemia. This accords well with the generally accepted notion that galactose is a poorer glycogen former than glucose. Similarly, the failure of moderate amounts of fructose to alter markedly the blood sugar level is attributed to a very rapid conversion of this sugar into glycogen. Foster is therefore led to conclude that glycogen formation is the chief factor tending to prevent alimentary hyperglycemia.

In connection with certain studies on the utilization of carbohydrates in experimental derangements of the liver, it appeared desirable to determine in a fairly large number of animals the tolerance for fructose, glucose, and galactose. For this purpose twenty healthy dogs, weighing between 6 and 20 kilos, were selected. In each case, the tolerance tests were preceded by a fast of about 20 hours. The blood was obtained by cutting one of the marginal veins of the ear. After collecting an initial blood specimen, the sugar, dissolved in about 200 cc. of water,

was given by mouth (3 gm. per kilo of body weight). Following the sugar administration, additional blood samples were collected at definite intervals. In determining blood sugar, we used the method of Folin and Wu (4). The glucose was Merck's c.p. preparation. Both the levulose and galactose were Pfanzstiehl's c.p. special grade.

In all the animals the tolerance for levulose, as determined by blood analyses, was better than either that for glucose or galactose. With but a single exception, galactose produced a more pronounced hyperglycemia than dextrose. These findings are therefore more comparable to those obtained by Foster in human subjects than to the results reported by Folin and Berglund.

For the purpose of economizing space, the data of but one experiment are given in detail (Table I). In addition, the averages obtained on the twenty dogs are presented in Table II. Results of other tests on individual animals are included in the data recorded in Tables III and IV.

In the present investigation, little attempt was made to determine the nature of the sugars in the circulation following the administration of the monosaccharides. It is to be recalled that Folin and Berglund were able to demonstrate levulose in blood plasma 20 minutes after the ingestion of this carbohydrate. However, the "sugar" appearing in the urine after fructose feeding did not give the tests for levulose. Folin and Berglund therefore suggest that the glycogenesis was due not to levulose, but to reducing decomposition products of levulose. Our results point to the same conclusion. In not a single instance did we obtain well defined tests for levulose in the urines of the normal dogs. Still more interesting are the findings with respect to the glycogenesis following galactose ingestion. In these experiments, as much as one-third of the total sugar administered, and in a few cases even more, was excreted in the urine during the 24 hours following the test. Nevertheless, the urines contained relatively little or no galactose. In only two cases were we able to establish the presence of appreciable amounts of this sugar (mucic acid and osazone tests).

Folin and Berglund have called attention to the remarkable effect of dextrose in increasing the retention and utilization of galactose. In one of their experiments, the administration of

100 gm. of the latter was followed by the excretion of 5,685 mg. of "extra" sugar, whereas the ingestion of 100 gm. of dextrose together with 100 gm. of galactose resulted in the elimination of less than one-tenth this amount; namely, 371 mg. This phenomenon can be demonstrated even more directly. It can be

TABLE I.

*Tolerance for Fructose, Glucose, and Galactose in Dog 15.*

Male. Weight 12.9 kilos.

Time after ingestion.	Blood sugar per 100 cc.		
	Experiment 1. 38.7 gm. fructose.	Experiment 2. 38.7 gm. glucose.	Experiment 3. 38.7 gm. galactose.
min.	mg.	mg.	mg.
Before.	105	87	104
15	126	162	138
45	125	178	238
75	111	191	328
135	109	128	342

TABLE II.

*Average Tolerance for Fructose, Glucose, and Galactose in Twenty Dogs.*

3 gm. of the sugar per kilo of body weight.

Time after ingestion.	Blood sugar per 100 cc.		
	Fructose.	Glucose.	Galactose.
min.	mg.	mg.	mg.
Before.	95	97	95
15	111	156	151
45	114	194	233
75	110	179	283
135	101	130	240

shown that the blood sugar curve produced by galactose is invariably depressed if both galactose and glucose are fed. On examining the data in Table III, it will be seen that when galactose and dextrose are administered together, the hyperglycemia produced is not much more marked than that following ingestion of glucose alone.

The possible effect of an altered rate of absorption of galactose when the two sugars are fed together, must not be omitted from

TABLE III.

*Showing the Effect of Glucose and Fructose Ingestion on Galactose Tolerance in Dog 9.*

Male. Weight 8.2 kilos.

Time after ingestion.	Blood sugar per 100 cc.				
	24.6 gm. fructose.	24.6 gm. glucose.	24.6 gm. galactose.	24.6 gm. galactose plus 24.6 gm. glucose.	24.6 gm. galactose plus 24.6 gm. fructose.
min.	mg.	mg.	mg.	mg.	mg.
Before.	104	89	99	106	107
15	127	135	154	126	187
45	138	185	270	149	274
75	136	178	370	180	344
135	120	102	226	235	240
255			110	115	

TABLE IV.

*Showing the Effect of Glucose and Fructose Ingestion on Galactose Tolerance in Dog 20.*

Male. Weight 9.8 kilos.

Time after ingestion.	Blood sugar per 100 cc.				
	29.4 gm. fructose.	29.4 gm. glucose.	29.4 gm. galactose.	29.4 gm. galactose plus 29.4 gm. glucose.	29.4 gm. galactose plus 29.4 gm. fructose.
min.	mg.	mg.	mg.	mg.	mg.
Before.	92	98	95	92	90
15	111	126	146	156	155
45	130	165	200	200	210
75	112	162	288	210	254
135	110	160	316	242	300
255			95	97	103

consideration. If for a given period only half as much galactose were to enter the circulation, one might expect a slower increase in the blood sugar than would occur if the absorption rate were

twice as rapid. However, any objection based on this assumption is untenable because if the absorption rate were the important factor, levulose, when fed with galactose should exert an even greater effect in depressing galactose hyperglycemia. This does not occur. In most experiments, the ingestion of levulose together with galactose was followed by as great an increase in the blood sugar as in the feeding experiments with galactose alone. It is to be admitted, however, that in a few cases, levulose depressed to a slight extent the galactose tolerance curve.

MacLean and de Wesselow (5) explain the appearance of the normal sugar tolerance curve by assuming that the carbohydrate storage mechanism comes into action at about a threshold of 0.16 to 0.17 per cent blood sugar. This view finds support in Foster who postulates a stimulation of the glycogenic function. Probable though this may be, there is actually little direct evidence to show that glycogen formation would account for the removal of most of the excess sugar from the circulation. Since the liver is the main site of glycogen synthesis, almost complete destruction of the liver parenchyma should result in a marked carbohydrate intolerance. As will be shown in a forthcoming publication, this does occur to some extent, but the increased hyperglycemia and glycuresis under these conditions account for only a fraction of the absorbed sugar. One might speculate on the possibility that in liver derangements, other tissues assume a greater share of the burden of glycogen formation and storage. There are, however, indications that the maintenance of the blood sugar within certain concentrations is under hormone control and that this mechanism is stimulated to greater activity by excessive amounts of glucose.

We believe that the results of our feeding experiments with galactose plus glucose lend some support to the "stimulation" theory. The accumulation of a certain concentration of glucose in the blood seems to provoke a more rapid rate of sugar removal. When galactose alone is fed, as shown both in the experiments of Foster and in our own experiments, the drop in blood sugar does not begin until after it has reached a relatively high level (240 to 290 mg. in Foster's experiments; 240 to 440 mg. in ours). An explanation that may perhaps be offered is that galactose, as such, does not provide as efficient a stimulus as does dextrose.

The eventual break in the curve is due partly to the excretion of some of the sugar and in part, perhaps, to the accumulation in the blood of some of the transformation products of galactose, including possibly at least one of the isomeric forms of glucose. The mechanism concerned with the removal of sugar from the

TABLE V.

*Showing the Effect of Glucose and Fructose on the Glycogenesis following Galactose Ingestion.*

Dog No.	Weight. kg.	Sugar.	Urine volume per 24 hrs. cc.	Urine sugar per 24 hrs. gm.
29	10.4	31.2 gm. fructose.	225	1.08
		31.2 " glucose.	230	0.77
		31.2 " galactose.	330	14.72
		31.2 " plus 31.2 gm. glucose.	395	9.32
		31.2 gm. galactose plus 31.2 gm. fructose.	445	14.70
30	10.5	31.5 gm. fructose.	730	0.98
		31.5 " glucose.	1,050	1.36
		31.5 " galactose.	1,125	10.80
		31.5 " plus 31.5 gm. glucose.	435	7.83
		31.5 gm. galactose plus 31.5 gm. fructose.	570	9.34
31	8.3	24.9 gm. fructose.	480	0.99
		24.9 " glucose.	500	1.79
		24.9 " galactose.	430	9.76
		24.9 " plus 24.9 gm. glucose.	475	5.65
		24.9 gm. galactose plus 24.9 gm. fructose.	205	10.25

circulation would thus be provided with the necessary stimulus. That this is not altogether a vague speculation, is supported by our findings that in the presence of sufficient glucose excessive hyperglycemia due to galactose is prevented.

The urinary sugar was determined by Benedict's method (6). The data in Table V show that the ingestion either of glucose or

of fructose (3 gm. per kilo of body weight) results in a slight glycuresis. In the case of galactose, on the other hand, the glycosuria is very marked, more than one-third of the ingested sugar being excreted. When dextrose is fed together with galactose, less sugar is excreted than when only galactose is given. It is to be noted that the diminished glycuresis under these conditions is associated with a lowered blood sugar level.

#### SUMMARY.

When administered to dogs, fructose is less effective than glucose in producing alimentary hyperglycemia. On the other hand, the feeding of galactose results in a very marked increase in the blood sugar concentration.

Following the ingestion of fructose and galactose, the urine contains reducing substances other than these monosaccharides.

The presence of sufficient glucose in the circulation prevents the excessive hyperglycemia due to galactose.

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## OBSERVATIONS ON THE DETERMINATION OF BLOOD UREA.

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For the determination of urea in the blood, it has been customary in this laboratory to use finely ground soy bean meal added to whole blood. At one time, when no fresh soy beans were available, an extract of jack bean meal, made according to Folin's (1) directions, was used for class work in the determination of urea in beef blood. The results obtained in this instance were markedly lower than those which we had previously found (using soy bean meal) for the urea content of the blood of this species. Indeed, 1 cc. of the dilute (1:20) extract of jack bean powder added directly to the blood yielded a urea nitrogen for beef blood amounting to only 30 per cent or less of the total non-protein nitrogen, while with fresh powdered soy bean a figure representing approximately 50 per cent of the total non-protein nitrogen was obtained. Preliminary trials showed that using either the jack bean extract or the powdered soy bean, it was possible to recover urea added to the blood quantitatively and that ammonia or urea contained in the soy bean meal did not account for the difference in the results.

A systematic investigation of the cause of the difference by the two procedures was therefore undertaken. It was found early in the work that the differences apparently depended upon the quantity of soy bean used, either in solid form, or in the form of extract, and it was possible to obtain a graded series of urea figures from the same blood, by using extracts of different concentrations.

Experiments showed that 0.5 cc. of a 5 per cent extract of soy bean meal, made by shaking the meal with permutit and 15 per cent alcohol according to Folin's directions for jack bean extract

(1), was the minimum amount which was certain to yield complete recovery of 50 mg. of urea added to 100 cc. of blood. We commonly employed 1 cc. of a 5 per cent extract of soy bean, which yielded results practically identical with those given by 0.5 cc.

For comparison with the dilute extract we used either a small amount of soy bean meal or a concentrated extract of soy bean. 0.5 gm. of soy bean meal yields no detectable ammonia when incubated alone or with pure urea solutions, but it results in much higher blood urea figures than does a dilute extract. Amounts of meal greater than 1 gm. yield appreciable amounts of ammonia nitrogen and, in an attempt to obtain a maximum blood urea figure, we made 50 per cent extracts of the meal, which were entirely ammonia-free. These were made by stirring the proper amount of meal with permutit and dilute alcohol (15 per cent) for about 3 minutes in centrifuge tubes, centrifuging, and pouring the thick supernatant fluid through coarse cheese-cloth. Up to 5 or 6 cc. of such an extract incubated for 10 minutes with pure urea solutions, gave theoretical figures for the nitrogen just as did the dilute extract.

These large amounts of concentrated extract gave a somewhat greater increase in apparent blood urea than did 0.5 gm. of meal, and they were used in most of this work. However, they do not retain their activity for more than a few hours and for comparison with the dilute extracts 0.5 gm. of meal, which yields no appreciable ammonia, can be used to equal advantage.

5 per cent extracts of fresh jack bean, prepared in the same way, yielded approximately the same figures from beef blood as the 5 per cent extracts from the soy bean. Concentrated jack bean extracts, however, gave less of an increase over the figure obtained with dilute extracts than did concentrated extracts of soy bean. Since, according to Mateer and Marshall (2), the jack bean contains over fifteen times as much urease as the soy bean, our 5 per cent jack bean extracts should have contained more urease than our 50 per cent soy bean extracts. It would thus seem that the high figures for urea in blood obtained with concentrated extracts were not due simply to a high urease concentration. In order to test this question we have made a few determinations of the urease content of the soy and jack bean extracts employed in the urea determinations in blood.

We followed the method of Van Slyke and Cullen (3), allowing 1 cc. of the urease to act upon 5 cc. of a 6 per cent urea solution containing buffer salts, made according to their directions. The action was allowed to proceed at 20°C. for 15 minutes, when the alkali was added and the tubes were aerated. We used 15 cc. of 0.1 N HCl in the receiving tubes and determined the ammonia by titration. It was found that under these conditions the 50 per cent soy bean extract was able to convert practically ten times as much urea as the 5 per cent soy bean extract, whereas the 5 per cent jack bean extract hydrolyzed from 7.5 to 8 times as much as the 5 per cent soy bean, or very nearly as much as the concentrated soy bean extract. Thus the ammonia formed by the action of the 5 per cent soy bean extract under these conditions neutralized an average of 1.62 cc. of 0.1 N HCl; that from the 50 per cent soy bean extract 15.8 cc.; and that from the 5 per cent jack bean extract 12.57 cc. The 5 per cent extracts of jack and soy beans, nevertheless, gave the same urea figures in blood, while 1 cc. of the 50 per cent extract showed an increase of 2 to 3 mg. of apparent urea nitrogen over the figure obtained with dilute extracts.

These results are hardly conclusive, but they appear to show that the increase in apparent urea when concentrated soy bean extracts are used does not bear a direct relationship to the concentration of urease, but involves some other factor. It is possible that the results are due to a second enzyme, present in relatively low concentration in soy bean, so that its action is only noticeable when high concentrations of the bean are used. It would be of interest to investigate other urease-containing beans in regard to this point.

We also tried the effect upon blood urea figures of using a commercial urease preparation in different amounts (Hynson, Westcott and Dunning) in the form of tablets and found that it behaved in the same way as did soy bean extracts. To give their maximal effect the tablets must be either ground or dissolved before they are added to the blood. The difference between the apparent urea figure in beef blood obtained from one tablet and that from three tablets is approximately the same as the difference between the figures when 1 cc. of 5 per cent extract and 2 cc. of 50 per cent extract of soy bean are used.

In the determinations from 0.5 to 2 cc. of the blood were treated with 2 or 3 cc. of distilled water, 3 drops of acid phosphate buffer solution (1), and the urease; and the mixture was incubated at 45–50° for 10 minutes. Longer periods of incubation had no effect upon the ammonia obtained. After the period of incubation 4 drops of caprylic alcohol and about a gram of solid sodium carbonate were added and the mixtures aerated, first slowly, then as rapidly as possible, for about 30 minutes, using 2 cc. of 0.1 N HCl in the absorbing tubes. At the end of this time the blood tubes were disconnected and aeration of the receiving tubes was continued for 10 minutes to remove the greater part of the caprylic alcohol. The solutions were then washed into volumetric flasks, nesslerized, and read in a colorimeter against ammonium chloride standards. Parallel ammonium chloride and urea controls were frequently run in series with the blood. Fresh extracts were made up each day.

Table I shows a comparison of urea nitrogen figures in whole beef blood when dilute and concentrated soy bean extracts and soy bean meal were used. The increase with concentrated extract is somewhat greater when 1 cc. of blood is used in the determination than when 2 cc. are used. Duplicate determinations with the same urease solution yielded figures which showed close agreement.

Most of the human bloods examined also show a higher apparent urea content when concentrated extract is used, but the differences are not so great in most instances as with beef blood. The bloods analyzed were all obtained from hospital cases.<sup>1</sup> It will be seen from Table II that the increase in the urea figure due to concentrated extract is usually not so marked in the nephritic bloods. A case of bichloride of mercury poisoning, on the other hand, with very high urea nitrogen, shows an increase with concentrated extract of about 45 mg. per 100 cc. above the figure obtained with dilute extract. From the figures so far obtained, one could draw no conclusion as to the possible clinical significance of the substance yielding increased urea following use of greater quantities of soy bean.

<sup>1</sup> We are indebted to Dr. Wm. G. Lyle, Director of the Harriman Research Laboratory, Roosevelt Hospital, for these bloods.

TABLE I.

*Showing a Comparison of the Figures for Urea Nitrogen in Beef Blood When Small and Large Amounts of Soy Bean are Used for Hydrolysis of the Urea.*

Sample No.	Amount of blood used in determination.	Urea nitrogen per 100 cc. of blood.				
		With 1 cc. of dilute extract.	With 0.5 gm. of meal.	Increase in apparent urea nitrogen.	Added urea nitrogen recovered.	
	cc.	mg.	mg.	mg.	With 1 cc. of dilute extract.	With 2 cc. of concentrated extract.
2	2	8.1	13.4	5.3	24.0*	24.5
3	2	9.1	16.2	7.1	11.6	12.0
1	1	12.8	18.8	6.0	24.5*	24.5
1	2	10.5	17.2	6.7		
With 2 cc. of concentrated extract.						
1	2	8.1	15.6	7.5	8.6	8.8
2	2	8.8	15.0	6.1		
4	2	9.3	16.7	6.3	22.2*	21.7
5	2	9.8	15.8	6.0	10.5	9.2
6	2	8.1	12.8	4.7	12.0	11.1
7	2	9.6	16.0	6.3		
8	2	10.0	16.7	6.7		
8	2	9.8	16.0	6.1		
9	2	9.3	13.8	4.5		
10	2	10.1	16.7	6.6	25.0*	23.7
11	2	10.3	17.7	7.3	22.7*	21.0
1	1	10.0	20.7	10.7	22.0*	22.7
2	1	10.8	19.9	9.1		
3	1	10.6	19.7	9.1	22.2*	23.0
1	0.5	13.0	25.0	12.0		
With 0.5 cc. of dilute extract.						
10	1	12.5	24.2	11.7		
12	1	10.1	27.0	16.9		
13	1	11.7	21.0	9.2	21.2*	21.5
13	1	11.7	19.7	8.0		

\* Pure solutions.

TABLE II.  
*Showing a Comparison of Urea Nitrogen Figures in Human Bloods When Dilute and Concentrated Soy Bean Extracts Are Used.*

Sample No.	Urea nitrogen per 100 cc. of blood.			Diagnosis.
	With dilute extract.	With concentrated extract.	In urine with concentrated extract.	
	mg.	mg.	mg.	
1	92.0	92.0	0.0	Nephritis.
2	84.0	78.0	-6.0	"
3	37.5	39.0	1.5	"
4	40.5	42.5	2.0	"
5	16.3	19.2	3.0	" and syphilis.
6	26.7	30.7	4.0	"
7	81.0	85.0	4.0	"
8	72.5	79.0	6.5	"
9	17.7	21.2	3.5	Hypertension, mild nephritis.
10	12.5	15.0	2.5	Diabetes.
11	14.8	19.5	4.6	"
12	21.0	22.7	1.7	"
13	18.7	24.0	5.2	"
14	12.8	22.7	9.9	"
15	23.0	29.5	6.5	"
16	30.0	36.5	6.5	Uremia.
17	139.4	182.0	42.6	Bichloride of mercury poisoning.
18	15.0	18.7	3.7	Pneumonia.
19	13.1	17.7	4.6	"
20	8.7	14.6	5.8	Tuberculosis and pleurisy.
21	12.5	16.7	4.2	" of lung.
22	13.5	20.1	6.6	Acute bronchitis.
23	15.2	25.7	10.4	Bronchitis and tonsilitis.
24	27.7	34.5	6.7	Empyema; acute bronchitis.
25	14.5	20.7	6.2	Carcinoma of liver (died).
26	7.0	11.7	5.0	Large liver and spleen.
27	31.2	40.0	8.7	Cholelithiasis.
28	16.4	19.2	2.8	Ulcer and general stomach trouble.
29	10.1	15.0	4.8	Chronic gastritis.
30	12.1	18.7	6.6	Ulcer of stomach.
31	9.3	12.8	3.5	Hyperacidity of stomach, general asthenia.
32	9.8	15.0	5.1	Hypertension and slight cardiac hypertrophy.
33	16.2	28.7	17.0	Hypertension.
34	20.7	28.2	7.5	Aortic stenosis.
35	13.1	20.3	7.2	Hypertrophy of prostate.
36	13.1	18.7	5.6	Lethargic encephalitis.
37	14.7	20.1	5.3	Urethral calculi.

The substance responsible for the increase when greater concentrations are used is chiefly contained in the corpuscles. In plasma concentrated extracts gave only slightly higher figures than dilute extracts. The results in this connection are given in Table III. The increase is greatest when small amounts of corpuscles are used in the determinations.

TABLE III.

*Showing the Distribution of "Pseudo Urea" Nitrogen between Corpuscles and Plasma of Beef Blood.*

Character of fluid.	With dilute extract.		With concentrated extract.		Increase in apparent urea nitrogen.
	0.5 cc.	1 cc.	2 cc.	5 cc.	
	mg.	mg.	mg.	mg.	
Whole blood.		10.0	20.7		10.7
Corpuscles.		15.0	30.0		15.0
Whole blood.		10.6	19.7		9.1
Corpuscles.		10.3	25.2		14.8
Whole blood.	12.2			26.2	14.0
Corpuscles.	10.8			34.0	23.1
Whole blood.		10.3	17.7		7.3
Plasma.		11.0	10.5		0.5
Whole blood.		8.1	15.6		7.5
Plasma.		9.6	11.8		2.2
Whole blood.	11.0			18.7	7.7
Corpuscles.	11.7			33.0	21.2
Plasma.	10.5			13.8	3.3
Whole blood.	10.1			17.0	6.8
Corpuscles.	10.0			30.2	20.0
Plasma.	9.3			11.7	2.8

It was obviously of interest to determine whether the different results obtained with the different amounts of soy bean would also be found in protein-free filtrates. Table IV records the results of experiments on this question with beef and human blood. The difference in effect of concentrated extract is found to be very slight or negligible in beef blood filtrates obtained by heat

TABLE IV.

*Showing the Figures for Blood Urea Obtained with Dilute and Concentrated Urease Extracts with Whole Blood and Tungstic Acid Filtrates.*

Source of sample.	Material analyzed.	Urea nitrogen per 100 cc. of blood.			Diagnosis.
		With dilute extract.	With concentrated extract.	Increase with concentrated extract.	
		mg.	mg.	mg.	
Beef 1.	Blood.	8.8	15.0	6.1	Nephritic.
	Filtrate.	10.1	10.3	0.2	
" 2.	Blood.	9.3	13.8	4.5	" " "
	Filtrate.	8.2	8.6	0.3	
" 3.	Blood.	11.7	20.3	8.5	Uremia.
	Filtrate I.	12.5	13.8	1.3	
	" II.	10.7	11.3	0.6	
Human.	Blood.	26.7	30.7	4.0	Mercury poisoning.
	Filtrate.	27.0	29.6	2.6	
" "	Blood.	72.5	79.0	6.5	" " "
	Filtrate.	73.0	78.0	5.0	
" "	Blood.	30.0	36.5	6.5	Diabetic.
	Filtrate.	28.2	30.0	1.7	
" "	Blood.	34.0	81.5	47.5	Cholelithiasis.
	Filtrate.	17.6	19.0	1.4	
" "	Blood.	14.8	19.5	4.6	Hyperacidity of stomach.
	Filtrate.	13.3	15.6	2.3	
" "	Blood.	8.7	24.0	5.2	Ulcer of stomach.
	Filtrate.	13.8	17.6	1.7	
" "	Blood.	9.3	12.8	3.5	Chronic bronchitis.
	Filtrate.	10.6	12.1	1.5	
" "	Blood.	12.1	18.7	6.6	Aortic stenosis.
	Filtrate.	10.8	11.0	0.1	
" "	Blood.	31.2	40.0	8.7	Diabetes.
	Filtrate.	31.2	31.4	0.2	
" "	Blood.	10.1	15.0	4.8	" " "
	Filtrate.	10.2	11.8	1.6	
" "	Blood.	20.7	28.2	7.5	" " "
	Filtrate.	17.2	18.2	1.0	
" "	Blood.	23.0	29.5	6.5	" " "
	Filtrate.	21.5	22.5	1.0	

coagulation or tungstic acid precipitation. Filtrates from some human blood samples, however, show a definite increase with concentrated urease, though this is not so great as the increase found in the same samples of whole blood.

Experiments now being carried out upon trichloroacetic acid filtrates seem to indicate that a compound which reacts only with concentrated soy bean is present to a certain extent in these filtrates.

We have tried a few experiments to determine something of the nature of the compound responsible for the increased ammonia when greater amounts of soy bean are used.

Beef blood, coagulated by heat before addition of the enzyme, fails to show any increase in apparent urea after use of the concentrated urease extract.

Hydrolysis of the blood by heating with dilute acid or incubation at body temperature fails to increase the free urea.

The compound in beef blood responsible for the increase is not lost by dialysis of the blood in collodion bags against running water for periods of 20 to 24 hours. The dialyzed blood showed no urea when dilute soy bean extract was used, while the concentrated extract yields the equivalent of 5 to 6.5 mg. of urea nitrogen per 100 cc. of original blood.

The increase in apparent urea nitrogen with concentrated extract was not found in raw milk nor did the results of one experiment on rabbit muscle indicate that it was present in that tissue.

#### SUMMARY.

When the soy bean urease method is applied to whole blood, the urea obtained is partially determined by the amount of meal or concentration of extract employed. In beef blood the apparent urea content may be increased by 50 per cent when a concentrated soy bean extract is used, though either concentrated or dilute extract will recover added urea quantitatively. Many human bloods also show a marked difference in urea content in relation to the concentration of the enzyme used.

Filtrates from beef blood (obtained by heat coagulation or by the use of tungstic acid) show practically no variation in relation to the concentration of enzyme employed.

Tungstic acid filtrates from human bloods usually show some increase with concentrated extract, but in many instances this increase may be very slight.

The compound responsible for the increase when large amounts of soy bean are used is present chiefly, if not wholly, in the corpuscles. It does not diffuse through collodion. Incubation, heat coagulation, or heating with dilute acid failed to increase the true urea content of whole blood. Moreover, the ability to produce this additional ammonia from the blood does not seem to be related to the urease concentration of the enzyme employed, but is evidently dependent upon other factors. It is possible that two enzymes are present in soy bean meal and crude extracts.

The "pseudo urea" found in a number of filtrates from human blood may represent a considerable proportion of the "undetermined" nitrogen in such bloods.

Our results indicate that the true urea content of blood is best represented by the urea figure obtained from blood filtrates. These figures are the same or slightly lower than the figures obtained from whole blood with small amounts of enzyme. Whether or not filtrates should be used exclusively for routine clinical work is a question which can better be answered when the significance of the "pseudo urea" in relation to pathological bloods is better understood. The results obtained with human bloods suggest very definitely that it would be worth while to study the apparent urea content of these bloods (using whole blood) with small and with large amounts of soy bean. We are attempting to study the question further.

In conclusion I wish to express my thanks to Professor S. R. Benedict for his advice throughout the work.

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## THE REACTION BETWEEN PROTEINS AND NITROUS ACID. THE TYROSINE CONTENT OF DEAMINIZED CASEIN.\*

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The action of nitrous acid upon proteins is not clearly understood. It is generally agreed that an important and primary reaction is the deamination of any free amino groups of the protein molecule with the liberation of their nitrogen, which may be measured quantitatively, as for example, in the well known apparatus of Van Slyke. The possibility of secondary reactions whose chemistry is less clearly understood has been suggested, but has not been carefully investigated.

A study of the properties of the various products obtained after the treatment of proteins with nitrous acid, the so called desamino or deaminized proteins, by different experimental procedures shows marked discrepancies. The greater number of products have been obtained by various modifications of the procedure described by Skraup and Hoernes (1). These investigators warmed casein with glacial acetic acid and by the addition of boiling water obtained a solution of casein in acetic acid. The solution was cooled and sodium nitrite was gradually added in the presence of a slow current of carbon dioxide. After standing for 4 hours, the mixture was heated on the water bath until foaming ceased. The same general procedure was adopted for other proteins studied by Skraup and his pupils except that, with soluble proteins, the preliminary heating with acetic acid was not necessary. Neither the duration of the heating on the water bath nor the temperature of the bath, factors which, in the light of the results to be reported in the present paper, seem of considerable importance, is given. Levites (2) employed a modification of this method. Casein was mixed

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\* Certain preliminary experiments were carried out by Mr. R. C. Corley in the Laboratory of Physiological Chemistry of the University of Illinois under the direction of the senior author (L). Desaminocaseins A, B, and C were prepared by Mr. M. S. Dunn in the same laboratory.

with an equal weight of solid potassium nitrite and, after addition of water, the mixture was heated on the water bath (temperature not stated) until the foaming ceased. After cooling, the desaminocasein was precipitated by the addition of acetic acid or by alcohol and ether. In a later method of Levites (3), casein was mixed with 10 per cent acetic acid and the mixture shaken in a machine until a fine emulsion was obtained. Sodium nitrite was then added slowly and the mixture was heated at 40° until foaming ceased. Dunn and Lewis (4) added glacial acetic acid drop by drop to a suspension of casein in water, and the mixture was vigorously stirred for 1½ hours. At the end of this period solution of the casein was obtained and sodium nitrite was added drop by drop with continued stirring for 1½ hours. The product was allowed to stand at room temperature (in some cases at 35°) for 18 hours and then filtered off, washed, and dried. The yields of desaminocasein obtained by this method were larger than those reported by Skraup or Levites. Other investigators (5 to 11) have used the original method of Skraup and Hoernes or very slight modifications of it. Treves and Salomone (6) worked at 0°, room temperature, and at 34–40° and employed hydrochloric acid in place of acetic acid.

Dunn and Lewis (4) were unable to determine the presence of any free amino nitrogen in the desaminocasein prepared by them. Herzig and Lieb (12), on the other hand, observed that the desamino proteins prepared by them according to the procedure of Skraup and Hoernes (1) contained as much free amino nitrogen as the original proteins themselves and concluded that their results were to be explained by a hydrolysis of the molecule with a consequent formation of new amino groups. In view of the fact that the proteins were heated for some time in the presence of relatively strong acetic acid and sodium nitrite, such an explanation of their results appears probable. Dunn and Schmidt (13) found no free amino nitrogen in casein which had been deaminized at 0°.

In Table I are collected the data which concern two important protein reactions, the biuret and Millon's, for the various deaminized proteins reported in the literature. Marked variations in the properties of the same protein prepared by different investigators are evident. Millon's reaction is reported positive in all desamino proteins prepared by the first method of Levites (2), in which the protein is warmed with sodium nitrite, but not in the presence of acetic acid. With the exception of the product of Dunn and Lewis (4), in the preparation of which heat was not employed, no other desamino proteins react positively in this test. Similar differences are seen in the biuret reaction.

In view of these facts, a study has been made of the tyrosine content of desaminocasein prepared by the various methods described. It seemed probable that the alleged destruction of

TABLE I.  
*Properties of Deaminized Proteins as Prepared by Various Methods Described  
in the Literature.*

Protein.	Temperature.	Biuret reaction.	Millon's reaction.	Observer.
	°C.			
Casein.	Water bath.	Negative.	Negative.*	Skraup and Hoernes (1).
"	" " †	Positive.	Positive.	Levites (2).
"	40	Doubtful.	Negative.	" (3).
"	20	Positive.	Positive.‡	Dunn and Lewis (4).
Gelatin.	Water bath.†	"		Levites (2).
"	40	"		" (3).
"	Water bath.	" §		Skraup (5).
Ovalbumin.	0, 20, 34	Doubtful.	Negative.	Treves and Salomone (6).
"	Water bath after some hours at room temperature.	"	"	Skraup and Kaas (7).
"	35-40	Negative.	Doubtful.	Schiff (8).
"	Water bath.†	Positive.	Positive.	Levites (2).
Vitellin.	40	Doubtful.	Negative.	" (3).
Gliadin.	40	Weak or negative.		" (3).
Serum globulin.	Water bath.	Doubtful.	Doubtful.	Lampel (9).
Edestin.	" "			Traxl (10).
Peptone (Witte).	Room.	Negative or very weak.	Negative.	Shrötter (11).

\* No tyrosine could be isolated on hydrolysis.

† Heated on water bath with potassium nitrite and cooled before the addition of acetic acid.

‡ Tyrosine was isolated by crystallization from the products of acid hydrolysis.

§ Deaminized gelatin was reported by Skraup to give as strong a biuret reaction as gelatin; the color, however, was observed to be more reddish than with normal gelatin.

|| The absence of tyrosine was established by direct determination.

tyrosine<sup>1</sup> might be a secondary reaction not concerned with the primary reaction of the deamination of the free amino groups, and that the decreased percentages of amino-acids, other than lysine, e.g. arginine and histidine, reported in deaminized proteins (1, 9, 14) might be similarly explained. The results obtained have been in confirmation of this theory and have shown that *treatment with sodium nitrite and acetic acid after deamination has been completed, may result in the partial destruction of the tyrosine, the extent of the destruction being proportional to the time of action of the reagents and the temperature.*

#### EXPERIMENTAL.

Casein was prepared by the method of Van Slyke and Bosworth (15) except that the final treatment with ammonium oxalate was omitted. Two different samples of casein were used in the deamination experiments in Table II. Desaminocaseins A, B, and C were prepared from casein 1, while all the other desaminocaseins were prepared from casein 2. As far as possible the methods of Skraup and Hoernes (1) and Levites (2, 3) were followed exactly. It was assumed that by water bath temperature a boiling water bath was meant, and the reactions were carried out at this temperature in Skraup's (1) and Levites's (2) first method. The time required in the heating until the cessation of foaming varied somewhat in the different experiments and as the importance of the time element was not fully recognized at first, no careful record of this was made. However, the variation in the period of heating was not markedly different in preparations made by the same method.

In the preparation of desaminocaseins N and O, the temperature was kept as low as possible, between 0 and 3°. 30 per cent sodium nitrite solution was cooled to 0° and the casein suspended in it with vigorous shaking. Glacial acetic acid which had been cooled as much as possible without obtaining solidification was added and the mixture kept at 0-3° in an ice-salt bath. The

<sup>1</sup> "Von den Monoaminosäuren ist zu sagen dass ihr Gehalt quantitativ wahrscheinlich unverändert bleibt, mit Ausnahme des Tyrosins, das bei allen Desamidoproteinen fehlt" (3). Dunn and Lewis (4), however, isolated tyrosine from the products of hydrolysis of desaminocasein prepared by them.

reaction was allowed to proceed at this temperature for 5 hours in the case of desaminocasein N and for 7 hours with desaminocasein O. Inasmuch as Dunn and Schmidt (13) have shown that deamination of casein is complete under these conditions in 1 hour, it was considered that ample time for complete deamination was afforded.

Tyrosine was determined according to the method of Folin and Looney (16). The colorimetric method for the determina-

TABLE II.  
*Tyrosine Content of Deaminized Casein Prepared by Various Methods.*

Protein.	Tyro-sine. per cent	Methods of preparation.
Casein 1.....	5.77	
Desaminocasein A.....	3.90	Dunn and Lewis (4).
"    B.....	4.54	" " " (4).
"    C*.....	5.39	Same as A18 (Dunn and Lewis, 4).
Casein 2†.....	5.94	
Desaminocasein D.....	3.98	Levites (2).
"    E.....	2.79	" (3).
"    F.....	3.28	" (3).
"    G.....	3.50	Skraup and Hoernes (1).
"    H.....	4.18	Dunn and Lewis (4).
"    L.....	3.59	" " " (4).
"    M*.....	4.37	Same as A18 (Dunn and Lewis, 4).
"    N.....	5.28	Lewis (see text).
"    O.....	5.05	" (" " ).

\* These caseins were filtered off as soon as they precipitated in contact with the acetic acid and sodium nitrite. At this time only about one-half of the free amino nitrogen had reacted. Cf. Dunn and Lewis (4), p. 333.

† All the desaminocaseins which follow in the table were prepared from this sample of casein.

tion of tyrosine by the use of Folin's phenol reagent has been criticized on the basis of non-specificity (17). In the modification of Folin and Looney, the tryptophane, the behavior of which in the reaction has been questioned, is precipitated by mercuric sulfate in the presence of sulfuric acid. In a recent comparison of the methods for the determination of tyrosine, Fürth and Fleischmann (18) have concluded that bromination of the products of hydrolysis of proteins after removal of the substances

precipitated by phosphotungstic acid gave results for tyrosine most nearly correct. They obtained 5.2 and 5.3 per cent of tyrosine in casein by this method. This compares favorably with the figures, 5.36 and 5.32 per cent reported by Folin and Looney (16) for the tyrosine content of casein by their new method. We have obtained slightly higher results, 5.77 and 5.94 per cent for the casein used in the present investigation.

It is to be borne in mind, however, that the method of Folin and Looney, as well as most other methods for the estimation of tyrosine, except the direct determination by isolation of the pure substance, which is tedious and inaccurate, depends upon the reaction of a phenol nucleus. Hence in the present series of experiments, although the results are for convenience reported as tyrosine, they may best be interpreted as significant of the phenol groups which have not reacted with the nitrite-acetic acid mixture in such a way as to render no longer possible the reactions characteristic of a phenol. It is probable that *p*-hydroxyphenyllactic acid, a possible product of the simple deamination of tyrosine, would react as would tyrosine and its presence in a deaminized protein instead of tyrosine would make it appear that the tyrosine content was not altered. Hence results which show an actual diminution of the group responsible for the reaction of Folin are significant as indicating an alteration of the phenol ring, so that it no longer reacts positively, and thus indicate a change in the tyrosine content.

The results obtained for the tyrosine content of the deaminized caseins prepared by the various methods discussed are presented in Table II. It will be noted that there is a wide variation in the percentage of tyrosine, from 5.39 (desaminocasein C) and 5.28 (desaminocasein N) to 2.79 (desaminocasein E) per cent. The percentages of tyrosine which differed least from that of the original casein were observed in desaminocaseins, in the preparation of which the deamination had been carried out at low temperatures (desaminocaseins N and O), or in those which had been removed from contact with the nitrite and acid shortly after the reaction had begun (desaminocaseins C and M) and before they were completely deaminized. Moreover, desaminocasein N, which was allowed to remain in contact with the acetic acid-nitrite mixture at 0° for 5 hours, contained slightly more

tyrosine than a similar preparation (desaminocasein O) which was allowed to react for the longer period of 7 hours. The desaminocasein D prepared by the first method of Levites (2), in which the protein was not heated after the addition of the acetic acid, had changed less in its content of tyrosine than had the desaminocaseins prepared by methods in which the proteins were heated in acid solution (desaminocaseins E, F, and G). Finally, desaminocasein prepared according to the method of Dunn and Lewis (4), in which no heating was employed, but which stood at room temperature for a considerable period of time (desaminocaseins A, B, H, and L), showed losses of tyrosine which varied, being greatest with desaminocasein L and least with desaminocasein B. With one exception (desaminocasein L), the tyrosine content of all these products was higher than that of deaminized caseins prepared according to methods of Levites and Skraup. These experiments served to demonstrate that it was possible to deaminate casein without a marked alteration in the tyrosine content of the product as compared with the original casein.

In order to supply further experimental evidence in support of the view that the destruction of tyrosine was a secondary reaction, not concerned with the removal of the free amino groups of the protein, further experiments were carried out with desaminocasein B (see Table II), which, by the Van Slyke procedure, had been shown to contain no free amino nitrogen. 10 gm. of this completely deaminized casein were suspended in 100 cc. of 30 per cent sodium nitrite and 50 cc. of glacial acetic acid and allowed to react at different temperatures for varying periods of time. The insoluble products of the reaction were filtered off, washed, dried, and analyzed for tyrosine. The results of the analyses (Table III) show a loss of tyrosine which became increasingly greater as the temperature was raised from 20 to 100°. Thus, in Experiment 5, in which the reaction proceeded for 24 hours at 20°, the product obtained contained 3.33 per cent tyrosine, or about 73 per cent of the amount present in the original desaminocasein B. In Experiment 8 the mixture was allowed to react at 20° until violent foaming ceased ( $4\frac{1}{2}$  hours). The temperature was then raised to the boiling point for 1 hour. The resulting desaminocasein contained only 45 per cent of the amount of tyrosine originally present in the desaminocasein. At low tem-

peratures (Experiment 9) in a period of 72 hours, the change in tyrosine content was comparable with that observed at room temperature in 24 hours. Inasmuch as these changes in the tyrosine content were obtained by further treatment with the acetic acid-nitrite mixture of a casein already completely deaminized (as shown by the Van Slyke method) it is evident that the alteration of the tyrosine content in the deamination of proteins is not concerned with the deamination, but with secondary reactions, the extent of which are dependent upon the time and temperature of the reaction.

TABLE III.

*Effect of Nitrous Acid (Sodium Nitrite and Acetic Acid) on the Tyrosine Content of a Completely Deaminized Casein.*

Experiment No.	Temperature. °C.	Time. hrs.	Tyrosine.
			per cent
Original desamino-casein.			4.54
5	20	24	3.33
6	45-50 } 20 }	7 } 17 }	2.30
8	20 } 95-100 }	4½ } 1 }	2.08
9	0-5	72	3.28

The action of the acetic acid-nitrite mixture on pure tyrosine solutions was also studied (Table IV). A stock solution of pure tyrosine was prepared. To 25 cc. of this stock solution were added 5 cc. of 20 per cent sodium nitrite and 5 cc. of 50 per cent acetic acid. The solutions were thoroughly mixed and incubated 1 hour at the temperatures indicated. It was noted at the end of this period that the color of the solutions had changed, from very pale lemon to an orange, the depth in color increasing with the temperature of the bath. After cooling, the solutions were treated with the amount of concentrated sodium hydroxide which had been found in a blank determination to be necessary to al-

most exactly neutralize the acidity. The nearly neutral solutions were transferred to volumetric flasks and diluted to 50 cc., and tyrosine (phenol) was determined in aliquots. It was thought that the presence of unchanged nitrite or of the sodium acetate from the neutralization of the acetic acid might interfere with the determination. A control experiment was carried out in which 5 cc. of the nitrite were incubated at 40° for 1 hour with 5 cc. of acetic acid. The acidity was neutralized, 25 cc. of the stock tyrosine solution were added, and the analysis was made as before. Comparative Millon's tests were also made on the diluted solutions.

TABLE IV.  
*Effect of Nitrous Acid (Sodium Nitrite and Acetic Acid) on Solutions of Pure Tyrosine.*

Experiment No.	Temperature.	Tyrosine.*	Millon's reaction.
	°C.	mg.	
Control.			Strong.
1	0-5	17.8 17.2 .	"
2	20-22	14.7	Positive.
3	40-45	Color too weak to read.	Questionable.
4	75	No color.	Negative.
5	100	" "	"

\* For convenience in comparison the results are calculated in terms of tyrosine. It should be borne in mind, however, that the procedure would result in deamination of the free amino group of tyrosine, and that what is determined is the amount of phenol groups capable of reacting with the phosphomolybdic reagent.

In comparison with the control, little loss of tyrosine (phenol) was observed after reaction for 1 hour at low temperatures. The deamination of the tyrosine should have been complete during this period, according to the results of Dunn and Schmidt (13), who found that the amino group of alanine was completely removed in 18 minutes at 4°. At 20-22°, 14.7 mg. (calculated as tyrosine) were shown to be present, while at 40-45°, the color was too weak to permit of a comparison with the standard in the colorimeter. After the reaction at 75 and 100°, no color was observed. Qualitative Millon's tests gave results which ran parallel to the quantitative determinations.

It is not the purpose of the present paper to discuss the possible chemical changes which have resulted in the losses of tyrosine observed, but rather to call attention to the possibility of the destruction of tyrosine as a secondary reaction, dependent upon the time and temperature of the reaction. It is believed that the variation in the chemical properties and composition of the deaminized proteins described in the literature can be explained on the basis of variations in the time and temperature during the reaction with nitrite and acetic acid. We have demonstrated this to be true as far as concerns tyrosine. Presumably the same factors may be concerned with the reported destruction of amino-acids (1, 9, 14), other than lysine, in the deamination reaction.

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## A PRODUCT OF MILD ACID HYDROLYSIS OF WHEAT GLIADIN.\*

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In a previous paper (Vickery, 1922), it was shown that, by varying the strength of the acid, it was possible to obtain a picture of the rate of hydrolysis of wheat gliadin from the breaking of the first peptide bond to the complete decomposition of the protein into amino-acids. No evidence was obtained from the curves of the existence of stable complexes containing any large proportion of the total number of peptide bonds in the gliadin molecule. This result is quite different from that obtained with enzymes, for Frankel (1916) found that when vegetable proteins are digested with pepsin-hydrochloric acid only about 20 per cent of the peptide bonds were split. Trypsin, acting on proteins previously digested with pepsin, effected a cleavage of about 70 per cent of the peptide bonds. This is in accord with the older views of Kühne's school that a part of the protein molecule is resistant to hydrolysis. A difference obviously exists between hydrolysis by means of enzymes and hydrolysis by means of acids, as has already been suggested by Northrop (1921).

It may be well to recall the experimental basis upon which the ideas of protein structure current at the beginning of the present century rested.

Schützenberger in 1875 boiled coagulated egg albumin with 3 per cent sulfuric acid for about 2 hours. He observed a voluminous precipitate which separated on cooling the mixture. This material, which he termed

\* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C. The writer wishes to express his thanks to Dr. Thomas B. Osborne for his interest in the work, also for much helpful advice and criticism.

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hemiprotein, yielded on further hydrolysis with sulfuric acid the amino-acids, leucine and tyrosine, together with amorphous substances. The filtrate from the hemiprotein also yielded amorphous material.

Kühne in 1876 found that pepsin acting on proteins yielded a peptone which resisted further action of this enzyme. Trypsin acting upon this peptone yielded a complex resistant body together with a mixture of simpler substances, among which tyrosine and leucine were readily recognized. When trypsin was allowed to act upon the original protein a resistant peptone was produced as well as the simpler bodies tyrosine and leucine.

Kühne further found that when 0.25 per cent hydrochloric acid was allowed to act on egg albumin at 40° two main products were formed which he called antialbumate and hemialbumose. The former was obtained as a precipitate on neutralization. The latter together with hemipeptone passed into the filtrate. The continued action of acid upon antialbumate produced antialbumid, which was less soluble and more stable. This latter substance Kühne regarded as identical with Schützenberger's hemiprotein. It was readily produced by the action of boiling 3 to 5 per cent sulfuric acid on albumin; was readily soluble in alkali and reprecipitated from such solutions on neutralization, and was unacted upon by pepsin-hydrochloric acid (Kühne and Chittenden, 1883).

These and similar observations led Kühne to a conception of protein hydrolysis which may be briefly stated as follows. As a first step the protein is broken into bodies belonging to two distinct groups; *viz.*, the hemi and the anti groups. Subsequently smaller molecules of hemipeptone and antipeptone, respectively, are formed. No distinction was drawn by Kühne between the hydrolytic action of dilute acid, of pepsin, or of trypsin. The essential feature of his view was the postulation of the existence in the protein molecule of a more resistant anti group and less resistant hemi group.

Neumeister (1887) further elaborated Kühne's views.

Attention was directed during the last decade of the past, and first years of the present century, to the improvement of the methods for the separation of these products of protein hydrolysis. Kühne had used sodium chloride and ammonium sulfate under definite conditions to salt out certain of these bodies. Pick (1899, 1902) elaborated a scheme of fractional precipitation by means of partial and complete saturation with ammonium sulfate, Haslam (1905, 1907) a system of precipitations with 50 per cent alcohol and half saturated ammonium sulfate, Adler (1907; see also Borkel, 1903) a system involving ferric ammonium alum as a precipitating agent, and Birchard (1909; see also Levene, Van Slyke, and Birchard, 1910) a scheme involving the preparation of Siegfried's carbamino compounds of the proteoses through the use of carbon dioxide together with calcium or barium hydroxide solution.

Fischer's (1906) comment on these methods is pertinent. He says: "...the precipitation methods are not capable of yielding pure products from so complicated a mixture as that produced by the decomposition of

proteins, and on that account the various kinds of albumoses and proteoses with which the physiologists reckon, are to be considered by the chemist only as inseparable mixtures. The true object of the scientist working in this field must be the preparation of chemically defined homogeneous substances."

Zunz (1911), in a monograph on the proteoses, studied the various bodies prepared from Witte's peptone by the highly elaborated procedures of Pick, Haslam, Adler, and others. Armed with modern analytical and experimental methods he was able to show that these procedures led to the preparation of mixtures, complexes, or combinations of proteoses, of which only those produced by Pick's methods presented any constancy of composition. He stated, however, that there is nothing which allows us to affirm that the mixtures, complexes, or combinations of proteoses obtained by these methods exist as such in the products of the disintegration of proteins through the agency of pepsin. He believed that the proteoses represent true polypeptides containing more or less long chains of amino-acids, but it must not be forgotten that these preparations are not chemically defined substances, but groups, complexes, or combinations of proteoses which he showed can be further fractionated by adsorption methods using mastic or kaolin (Rona and Michaelis, 1907, *a* and *b*), or by ultrafiltration. Notwithstanding these criticisms of the precipitation methods, Heiduschka and Komm (1922, 1923) have recently carried out an elaborate fractionation of the products obtained by mild hydrolysis of keratin according to the methods outlined by Pick, Siegfried, and others.

It was inevitable that confusion in nomenclature and ideas should arise when the characterization of such highly complicated bodies as the proteoses depended almost entirely upon solubility in salt solutions of various definite concentrations. Nor were matters likely to be improved when so little distinction was drawn between different methods of hydrolysis. Nevertheless, there seems to have been a consensus of opinion that one relatively large portion of the protein molecule possessed a higher degree of stability than the rest of the molecule.

During my previous work it was observed that when gliadin was boiled with hydrochloric acid of concentrations between 0.2 and 4 N, a precipitate separated after boiling for a longer or shorter time according as the acid was weaker or stronger. This precipitate appeared in notable quantity when from 10 to 15 per cent of the nitrogen of the gliadin had been split off as ammonia, and increased in amount as amide hydrolysis proceeded to completion. Thereafter, as boiling continued, the precipitate diminished in quantity, very little remaining as a rule when 50 per cent of the

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peptide bonds had been hydrolyzed. According to Kühne's view this precipitate might consist of products derived from the so called anti group, in which case it should be relatively resistant to hydrolysis. On the other hand, taking into consideration the apparent connection between the formation of the precipitate and the progress of amide hydrolysis, the substance might possibly be simply gliadin from which amide nitrogen had been removed.

To obtain evidence regarding the relationship between the extent of amide hydrolysis of gliadin and the appearance of the insoluble material, 20 cc. quantities of a 5 per cent solution of

TABLE I.  
*Gliadin Containing 0.1658 Gm. of Nitrogen in 2.3 N HCl at 40 ± 1°C.*

Time.	Ammonia N.	Total N as ammonia N.	N in pre-cipitate.	Amino N in pre-cipitate.	Amino N in filtrate.	Free amino N. Sum of Columns 5 and 6.
hrs.	mg.	per cent	mg.	mg.	mg.	mg.
4	16.4	9.9	0.0			
7	27.2	16.4	64.1.	11.6	4.5	16.1
16	36.4	22.0	69.8	12.5	5.1	17.6
20	37.9	22.8	63.3	12.3	5.7	18.0
44	39.1	23.6	51.0	11.2	10.5	21.7
64	39.2	23.7	45.5	11.3	13.5	24.8
73	39.0	23.5	45.5	14.0	15.4	29.4
112	39.4	23.8	38.5	15.0	17.4	32.4

gliadin in 70 per cent alcohol were added to 80 cc. of 10.4 per cent hydrochloric acid, previously warmed to 40°C., and placed in an incubator maintained at 40 ± 1°C. Under these conditions the concentration of hydrochloric acid was 2.3 N. The precipitate, which had formed at the expiration of the desired time, was separated and washed by centrifuging, dissolved in 40 per cent acetic acid, and total as well as amino nitrogen determined. Ammonia and amino nitrogen were also determined in the liquid from which the precipitate had been removed.

Table I shows that the amount of nitrogen in the precipitate reached a maximum when amide hydrolysis was nearly complete and thereafter diminished. Peptide hydrolysis likewise occurred as shown by the increase in amino nitrogen in both precipitate and filtrate. Moreover, the proportion of amino to total nitrogen in the precipitate increased. This indicates that the insoluble

fraction undergoes continual change during hydrolysis and militates against the view that it is either gliadin from which amide nitrogen has been removed or that it is a markedly stable part of the gliadin molecule.

In the previous paper (Vickery, 1922), I showed that with boiling 0.2 N hydrochloric acid, amide hydrolysis of gliadin is nearly completed in 6 hours, while at the same time only from 5 to 6 per cent of the peptide bonds are split. Consequently, 100 gm. of air-dry gliadin, containing 16.43 gm. of nitrogen, were dissolved in 500 cc. of 70 per cent ethyl alcohol and poured into a solution containing 21.87 gm. of hydrochloric acid. The total volume, including 200 cc. of 70 per cent alcohol used for rinsing, was 3,000 cc., the concentration of acid 0.2 N, and the temperature 80°C. The flask was immediately connected to a reflux condenser, placed in a warm oil bath, the mixture heated to boiling within 20 minutes, and then maintained in gentle ebullition at 90–91°C. for 6 hours. The flask was then cooled to 40° within 15 minutes. The solution was thus heated approximately 6½ hours. The precipitate increased greatly in amount during the cooling operation. A dilute solution of sodium hydroxide was then slowly added with continuous stirring until the precipitate flocked out sharply and the supernatant liquid became clear. This precipitate was centrifuged off and washed once with water containing a little sodium chloride to prevent the formation of a colloidal suspension. It contained substances soluble in absolute alcohol which were removed by repeatedly suspending the precipitate in this solvent at room temperature until no further solid matter dissolved. The insoluble part, Preparation 1, when dried over sulfuric acid, ground, and finally dried in a vacuum, weighed 53.62 gm. and contained:

Preparation 1.	
	per cent
Moisture.....	1.76
Ash.....	0.16
Nitrogen.....	13.62
“ ash- and moisture-free.....	13.89

Preparation 1 thus contained 7.30 gm. of nitrogen or 44.5 per cent of the nitrogen of the original gliadin. 1 gm. required 20.7

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cc. of tenth normal alkali to obtain a faint, but permanent, alkaline reaction to phenolphthalein.

The straw-yellow alcoholic extracts (Solution 2) of the neutralization precipitate were united and evaporated under diminished pressure to dryness. The residue weighed 11.5 gm. and was dissolved in 50 per cent alcohol. Analyses of one-tenth of this solution (Solution 2) showed the whole to contain 1.185 gm. of nitrogen or 7.21 per cent of the nitrogen of the original gliadin. Of this, 0.035 gm. was free ammonia nitrogen doubtless derived from a small amount of ammonium chloride retained by the neutralization precipitate. The biuret, Millon's, and glyoxylic acid tests were positive. With picric acid a precipitate formed which dissolved on warming but reappeared on cooling, a reaction formerly considered to be characteristic of some proteoses.

The aqueous liquid, from which the insoluble product of hydrolysis had been removed, was concentrated under diminished pressure and made up with the addition of alcohol to 2,000 cc. One-tenth of this was removed for analysis (Solution 3). The whole solution contained 3.64 gm. of ammonia nitrogen and 3.953 gm. of non-ammonia nitrogen. The distillate (Solution 4) from the concentration of this liquid was found to contain 0.034 gm. of non-ammonia and 0.0305 gm. of ammonia nitrogen which had evidently been carried over during the distillation. This was not recovered. During the concentration a small amount of slimy material (Preparation 5) separated on the walls of the flask. It contained 0.0648 gm. of nitrogen.

The data in Table II give the distribution of nitrogen in the fractions thus obtained.

The remainder of Solution 3 was dialyzed until free of salts. The dialysate (Solution 6) contained nearly a gram of nitrogen other than ammonia of which about one-half belonged to peptides which could be precipitated by saturation of the solution with ammonium sulfate. Salts were removed from the filtrate from this precipitate by means of alcohol followed by treatment with a slight excess of barium hydroxide at boiling temperature. About one-half the nitrogen of the peptides now remaining in solution could be precipitated by phosphotungstic acid. This precipitate contained amino nitrogen amounting to about one-fifth of the total nitrogen. After hydrolysis with 20 per cent

hydrochloric acid for 24 hours this ratio was approximately doubled. The solution then yielded a precipitate with phosphotungstic acid, indicating the presence either of basic amino-acids or of some extremely stable peptide, such as the *L*-prolyl-*L*-phenylalanine of Osborne and Clapp (1907).

The peptides in the dialysate (Solution 6) which were precipitated neither by ammonium sulfate nor by phosphotungstic acid, contained approximately one-third of their nitrogen as amino

TABLE II.

*Distribution of Nitrogen in Fractions Obtained from 100 Gm. of Gliadin Containing 16.43 Gm. of Nitrogen when Hydrolyzed for 6 Hrs. with 0.2 N HCl at 90-91°C.*

Preparation.	Total nitrogen.		Free ammonia nitrogen.		Non-ammonia nitrogen.	
	gm.	per cent*	gm.	per cent*	gm.	per cent*
Preparation 1.....	7.30	44.45	0.0	0.0	7.30	44.45
Solution 2.						
Alcohol extract of Preparation 1.....	1.185	7.21	0.035	0.21	1.15	7.0
Solution 3.						
Aqueous mother liquor of Preparation 1.....	7.595	46.22	3.64	22.16	3.955	24.06
Solution 4.						
Distillate from Solution 3..	0.0645	0.39	0.0305	0.19	0.34	0.20
Preparation 5.						
Slimy material.....	0.0648	0.39			0.0648	0.39
Total.....	16.209	98.66	3.705	22.56	12.81	76.10

\* Per cent of total nitrogen present.

nitrogen. This ratio was raised to approximately three-quarters by hydrolysis with 20 per cent hydrochloric acid for 24 hours.

These data indicate the presence of relatively simple peptides in this dialysate and also that free amino-acids were present, if at all, only in extremely small amounts.

The distribution of the basic amino-acids in Preparation 1, as determined by Van Slyke's method, is given in Table III.

The best recorded analysis of gliadin by the Van Slyke method is one published by Osborne, Van Slyke, Leavenworth, and Vingrad (1915). This is quoted in Table IV, Column 2. In order

to compare the distribution of nitrogen in Preparation 1 with that in gliadin it is necessary to bear in mind that about a quarter of the nitrogen of the latter substance is amide nitrogen while only 4.5 per cent of the nitrogen of the former occurs in this form. Consequently, in Columns 3 and 4 this comparison is made in terms of the total nitrogen exclusive of the amide nitrogen.

It is clear that there are distinct differences between gliadin and Preparation 1 in the proportion of arginine, lysine, and non-

TABLE III.

*Van Slyke Analysis of Preparation 1. Data Corrected for Solubility of Bases. Total Nitrogen in Aliquots Used for Analysis Was 0.5312 Gm.*

Nitrogen.	I		II		Average.	
	gm.	per cent	gm.	per cent	gm.	per cent
Amide.....	0.0243	4.57	0.0340	4.52	0.0342	4.55
Humin in lime.....	0.0032	0.60	0.0023	0.48	0.0018	0.52
" " amyl alcohol....	0.0008	0.15	0.0009	0.17	0.0009	0.17
Cystine.....	0.0064	1.20	0.0080	1.50	0.0072	1.35
Arginine.....	0.0496	9.34	0.0496*	9.34*	0.0496	9.34
Histidine.....	0.0227	4.27	0.0197	3.71	0.0192	3.99
Lysine.....	0.0144	2.71	0.0148	2.78	0.0146	2.75
Amino in filtrate.....	0.3590	67.57	0.3606	67.87	0.3598	67.72
Non-amino in filtrate.....	0.0493	9.27	0.0445	8.38	0.0469	8.83
Total.....	0.5297	99.68	0.5244	98.70	0.5272	99.22

\* In Determination II a small amount of vapor was lost during the distillation. 0.0440 gm. of arginine nitrogen was found.

amino nitrogen of the filtrate from the bases (probably mostly proline nitrogen).

The total amount of arginine, histidine, and lysine nitrogen in 100 gm. of gliadin is given in Table V, together with the total amount of nitrogen of these three amino-acids found in the 56.6 gm. of Preparation 1 obtained from this amount of gliadin.

It is clear from these data that, within the experimental error, the entire amount of the lysine and approximately one-half the histidine and three-quarters of the arginine in gliadin are present in Fraction 1.

As a result of these analyses it is therefore quite certain that Preparation 1 does not represent gliadin from which amide nitrogen has been removed but which is otherwise unchanged.

To compare the rate of hydrolysis of Preparation 1 with that of gliadin, 1 gm. quantities were weighed out, 80 cc. of 2.5 N hydrochloric acid and, in order to make the conditions comparable with those under which gliadin was previously hydrolyzed (Vickery, 1922), 20 cc. of 70 per cent alcohol were added. The flasks were placed on an electric hot-plate under reflux condensers. Ebullition occurred within 10 minutes and the temperature of

TABLE IV.  
*Distribution of Nitrogen in Gliadin and Preparation 1 Calculated Free of Amide Nitrogen.*

Nitrogen.	Gliadin.	Gliadin exclusive of amide N.	Preparation inclusive of amide N.
	per cent	per cent	per cent
Amide.....	24.61*	0.00	0.00
Humin, insoluble.....	0.58†	0.78	
" in lime.....			0.54
" " amyl alcohol.....			0.18
Cystine.....	0.80	1.07	1.42
Arginine.....	5.45	7.31	9.78
Histidine.....	3.39	4.55	4.18
Lysine.....	1.33	1.78	2.88
Amino in filtrate.....	51.95	69.72	70.96
Non-amino in filtrate.....	10.70	14.36	9.25

\* We have repeatedly found the proportion of amide nitrogen on complete hydrolysis of our sample of gliadin to be 25.5 per cent. This figure is consequently used in these calculations.

† The humin nitrogen given here represents that in the total humin found in this analysis of gliadin.

the boiling mixture ranged from 94°, when boiling commenced, to 99° at the expiration of the longest hydrolysis periods. The free ammonia and amino nitrogen were determined as in the previous work. To determine the total peptide nitrogen for calculating the proportion of the peptide bonds hydrolyzed at various stages, 1 gm. of Preparation 1, containing 0.1361 gm. of nitrogen, was boiled with 20 per cent hydrochloric acid for 24 hours. The free amino nitrogen thus liberated was equal to 0.1028 gm. or 75.5 per cent of the total nitrogen. The experiments were made in duplicate. The data are given in Table VI and are plotted in Fig. 1 together with the data for the hydrolysis of gliadin under the same conditions.

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The two curves show that in the early stages there is but little difference between the rates at which the peptide bonds of Preparation 1 and of gliadin are broken by 2 N hydrochloric acid. Beyond 60 per cent of complete hydrolysis the rate at which Preparation 1 is hydrolyzed is somewhat slower than for gliadin, but no great difference is apparent when 80 per cent of the bonds have been broken. The curve for Preparation 1 becomes flat

TABLE V.

*Nitrogen of the Basic Amino-Acids in 100 Gm. of Gliadin and in 56.6 Gm. of Preparation 1 Obtained from 100 Gm. of Gliadin.*

	Gliadin. gm.	Preparation 1. gm.
Total N.....	16.43	7.303
Arginine N.....	0.895	0.682
Histidine N.....	0.557	0.291
Lysine N.....	0.218	0.200

TABLE VI.

*Rate of Hydrolysis of Preparation 1 by 2 N Hydrochloric Acid.*

Time. hrs.	Total N as amino N. per cent	Total peptide N as free amino N. per cent
0	0.0	2.2
1	3.5	13.7
4	3.8	35.2
10	4.3	60.9
12	4.3	62.5
20	4.5	70.7
24	4.6	71.6
34	5.0	78.0
45	5.1	88.0
76	5.6	88.9
88	5.4	88.5

after 45 hours, at which time 88 per cent of the bonds are broken, and subsequently rises very slightly. It seems probable, therefore, that Preparation 1 yields, on long hydrolysis with 2 N hydrochloric acid, a mixture containing relatively stable but simple peptides. In view of Osborne and Clapp's (1907) experience with the exceedingly stable dipeptide of proline and phenylalanine from gliadin, this is not surprising.

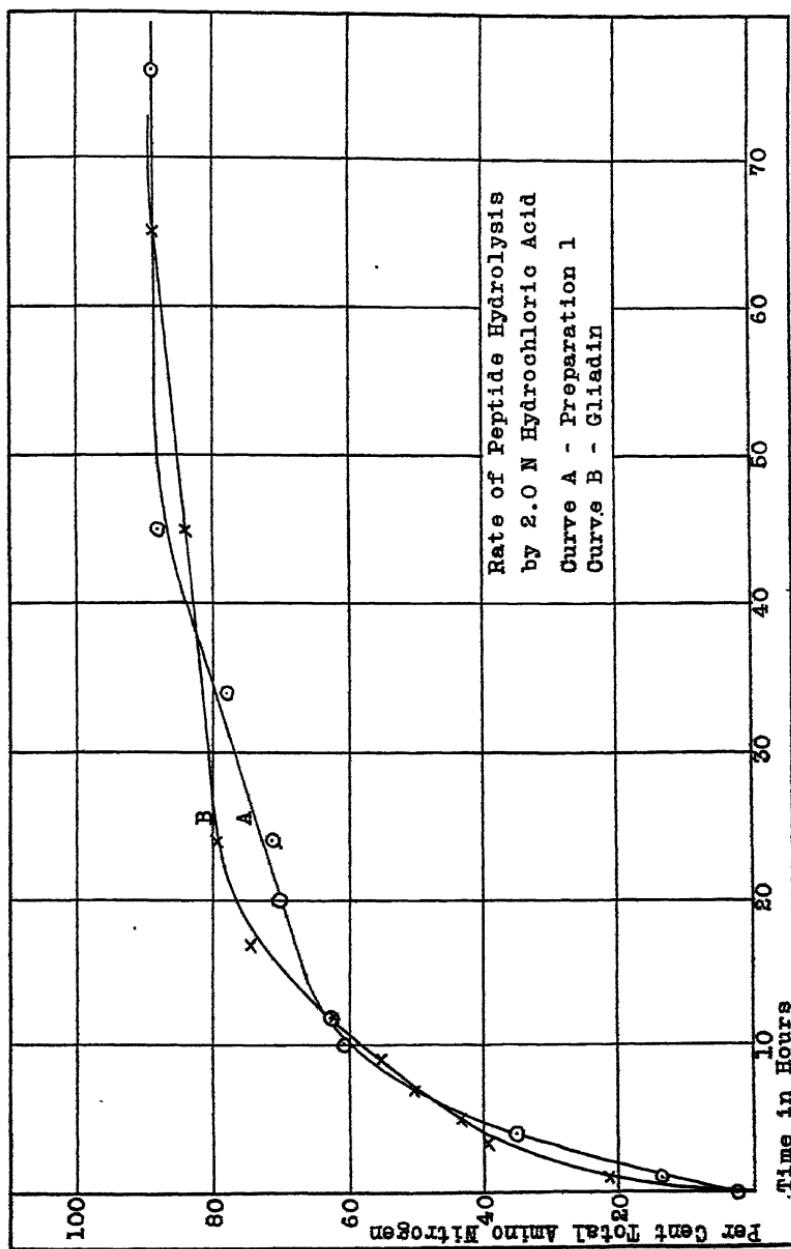


Fig. 1. Rate of peptide hydrolysis by 2 N hydrochloric acid of Preparation 1 and of gliadin.

The gradual evolution of ammonia, probably due to deamination, is also well shown by the data.

With respect to its content of arginine, lysine, and the non-amino nitrogen of the filtrate from the basic amino-acids, which is probably very largely proline nitrogen, the insoluble product obtained when gliadin is hydrolyzed with dilute acid at boiling temperature differs in constitution from that calculated for gliadin from which amide nitrogen alone has been removed. Moreover, when prepared under the conditions detailed above, this insoluble fraction contains all the lysine of the original gliadin. In its production, therefore, hydrolysis of peptide bindings as well as of amide bindings has played a part. Efforts to remove only amide nitrogen from gliadin, without simultaneously rupturing peptide bonds, have been unsuccessful. The rate at which about two-thirds of the peptide bonds of the above insoluble product are hydrolyzed is not materially different from that observed for gliadin. The view that it represents a portion of the protein molecule exceptionally resistant to hydrolysis consequently is untenable.

Whether or not this insoluble fraction of gliadin bears any real relation to the similarly insoluble hemiprotein of Schützenberger or the antialbumid of Kühne is difficult to determine. It is possible that if these investigators had had at their disposal a good method for following the progress of hydrolysis they would have found that these substances were not as stable towards acids as they assumed them to be.

It is highly improbable that this fraction of gliadin represents a definite chemical individual. As Zunz has pointed out, the separation of a precipitate from a partially hydrolyzed protein solution is no evidence that the configuration of the proteose complex so obtained necessarily represents the configuration in the original protein molecule. Two proteoses originating from entirely different parts of the protein molecule may both find their way into a neutralization precipitate and their presence may thus lead to wholly erroneous ideas of the constitution of the original protein.

A striking outcome of these experiments is the total lack of evidence that any *free* amino-acids were produced during the hydrolysis which involved the rupture of from 5 to 6 per cent

of the total peptide bindings of the gliadin molecule. No evidence was obtained to show at what stage of the hydrolysis free amino-acids appear, but even after boiling for more than 80 hours with 2 N hydrochloric acid the chart shows that over 10 per cent of the peptide bonds were still intact.

#### SUMMARY.

The results of this investigation do not support the old idea that hemi and anti groups exist in the protein molecule. Whether evidence of such could be obtained with some other protein than gliadin remains to be demonstrated. Under none of the conditions employed has it been possible to effect a differential hydrolysis of gliadin whereby the amide bindings only were broken. Although the hydrolysis of the amide bonds proceeds at a much more rapid rate than that of the peptide bonds these two reactions have been found always to take place simultaneously.

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## CHLORIDE AND CONDUCTIVITY DETERMINATIONS ON PLASMA.

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The purpose of this paper is to present studies upon the chloride content and the conductivity of the hirudinized plasma in health and in certain diseases, in which variations of chlorides are known to exist. *A priori* a certain parallelism between chloride analysis and conductivity measurements on plasma might be expected as already noted by Bugarszky and Tangl (1) who, however, demonstrated the depressing influence on conductivity exerted by proteins and other non-electrolytes (Oker-Blom, 2). The sodium chloride concentration and the cell volume of whole blood were also determined and from these data the chloride concentration of the corpuscles was calculated. The chloride concentration of whole blood is not given in the tables, but may be calculated from the other values.

The blood was taken from a vein at the elbow and allowed to drip from the needle into a 4 cc. centrifuge tube containing a little hirudin.<sup>1</sup> In order to prevent evaporation the tube was stoppered as soon as the necessary amount was obtained. All tubes were cleaned with steam in order to remove the least trace of electrolytes. After taking samples for determination of cell volume and for analysis of the whole blood, the plasma was separated from the remainder in a high speed centrifuge.

*Chloride determinations* were made by Bang's (4) micro method, modified in the way described in a previous paper (5). Results are expressed in terms of sodium chloride percentage.

*Conductivity measurements* were made with the ionometer<sup>2</sup> of Christiansen (6). This instrument is a voltmeter connected

<sup>1</sup> For the procedure of obtaining hirudin see Gram and Norgaard (3).

<sup>2</sup> Manufactured by Helweg-Mikkelsen, Ltd., Copenhagen.

with the house current (220 or 110 volts, d.c.). A magnetic shunt, which is adjusted before each determination, allows regulation for variations in the house current. The liquid to be examined is held in a U-shaped vessel (capacity  $\frac{1}{2}$  to 1 cc.) with bright platinum electrodes. By a suitable contact this vessel is inserted in series with the voltmeter and the degree of deflection

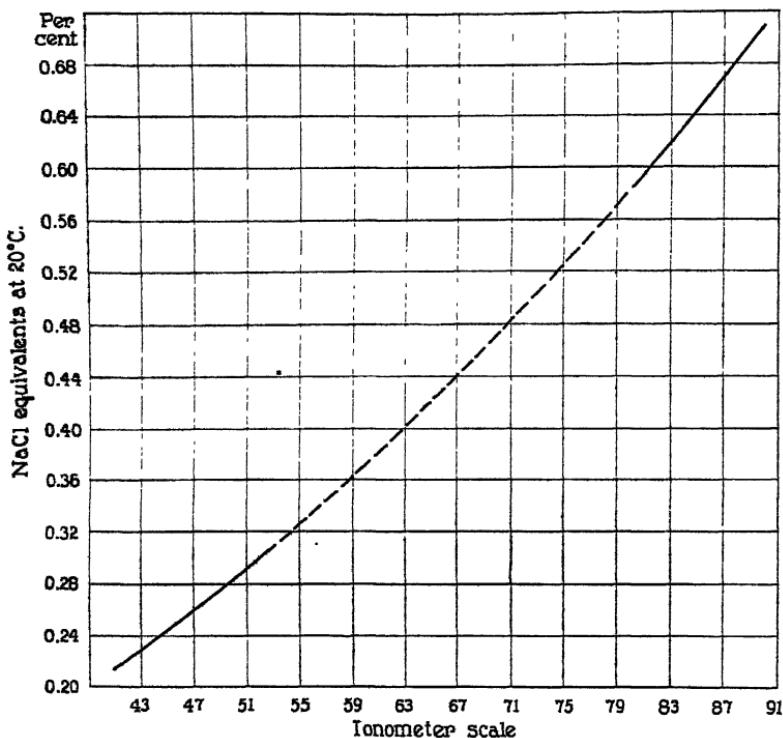


FIG. 1. Correction curve for ionometer (No. 1). 220 volts, d.c.

of the voltmeter serves as a measurement of the conductivity. In each case the mean of three readings was taken, the whole procedure, including the filling of tube and bath, taking less than 1 minute. There is not sufficient polarization under the conditions to affect the results. The temperature of 20°C. was chosen for the determinations, this being near the average temperature

of our laboratory. A vessel with water at the desired temperature will serve as a water bath during the short time taken by the examination.

The ionometer readings are expressed in this paper in sodium chloride equivalents; *i.e.*, the percentage of NaCl in aqueous solution required to give the same ionometer reading. The calibration of the ionometer against known aqueous sodium chloride solutions was repeated several times with identical

TABLE I.  
*Normal Individuals.*

No.	Age. yrs.	Sex.	Date.	NaCl equiv- alent of plasma.		NaCl titrated NaCl equivalent NaCl content of cor- puscles.	Cell volume. per cent
				per cent	per cent		
1	19	Male.	May 1	0.653	0.612	0.94	0.29 46.5
			" 16	0.684	0.622	0.91	0.28 46.7
2	37		" 6	0.653	0.615	0.94	0.31 47.5
			" 20	0.653	0.612	0.94	0.30 45.5
			Aug. 23	0.670	0.609	0.91	0.34 44.2
			Apr. 25	0.650	0.615	0.95	0.25 39.5
3	28	Female.	" 27	0.634	0.595	0.94	0.32 39.9
4	38		" 28	0.653	0.615	0.94	0.33 41.2
5	23		May 19	0.652	0.610	0.94	0.30 39.9
6	46	"	" 2	0.647	0.602	0.93	0.33 40.2
			" 18	0.652	0.620	0.95	0.30 38.0
7	20	"	Sept. 4	0.639	0.625	0.98	0.30 40.2
<u>Maximum.....</u>				0.684	0.625	0.98	0.34 47.5
<u>Minimum.....</u>				0.634	0.595	0.91	0.25 38.0
<u>Average.....</u>				0.653	0.613	0.939	0.304 42.4

results. The calibration curve for the instrument used is shown in Fig. 1. This method in our hands has given reliable results and has greatly facilitated conductivity determinations. Although by the method of bleeding we have employed, a slight shift in chlorides from corpuscles to plasma due to loss in CO<sub>2</sub> is to be expected we believe that the uniformity in our procedure of taking the blood may be assumed to have led to a relatively constant change in this respect.

The absence of electrolytes in our hirudin preparation has been controlled by examination of the dissolved product with the ionometer.

*The cell volume determinations* were made with a very finely graduated hematocrit described by us (3) in a previous paper. Complete transparency of the whole cell column was obtained in all cases, the mean of two observations being noted in each case.

#### *Chloride and Conductivity Determinations on Plasma.*

Our material is classified in the following groups: (1) Normal individuals; (2) pulmonary infections; and (3) diseases of the kidneys.

#### *Normal Individuals.*

Twelve specimens were obtained from seven persons.

The findings on normal plasma appear in Table I. The NaCl equivalents of the ionometer readings were in all cases slightly (averaging 0.041) higher than the sodium chloride percentages found by titration. The conductivity due to all electrolytes is expressed in terms of sodium chloride, but the protein influence on conductivity excludes the possibility of making the difference between titrated chlorides and the chloride equivalent of the conductivity a measure of non-chloride electrolytes; evidently, however, the amount of non-chloride electrolytes plays a small rôle in comparison with the chloride electrolytes. Rise and fall in the titrated chloride content was approximately proportionate to the corresponding variations in the ionometer equivalents. The average percentage of titrated chloride in these plasmas was found to be 0.613 per cent NaCl, agreeing quite well with the results of a previous series in which we (5) found 0.609 as the average of fifteen determinations. As the limits of the normal we find 0.595 and 0.625 per cent. The sodium chloride equivalents of the conductivity varied between 0.634 and 0.684 with an average of 0.653 per cent. The ratio

$$\frac{\text{Titrated NaCl}}{\text{NaCl equivalent of conductivity}}$$

varied between 0.978 and 0.907 with an average of 0.939.

TABLE II.  
*Pulmonary Injections.*

No.	Age.	Sex.	Date.	NaCl equivalent of plasma (titrated). per cent	NaCl content of cor- puscles. NaCl titrated NaCl equivalent per cent	Cell volume. per cent	Diagnosis.
1	10	Female.	May 22	0.628	0.542	0.86	41.1
			" 24	0.635	0.593	0.93	43.6
			" 29	0.628	0.591	0.94	41.2
2	65	Male.	Sept. 8	0.635	0.604	0.95	Lobar pneumonia.
			" 11	0.656	0.593	0.90	Bronchopneumonia.
			" 18	0.652	0.628	0.96	
3	22	"	May 9	0.629	0.589	0.94	"
			" 27	0.633	0.578	0.91	Pulmonary tuberculosis.
4	23	"	" 9	0.631	0.619	0.98	"
			" 26	0.631	0.608	0.96	
5	26	"	" 17	0.625	0.580	0.93	
6	26	"	" 17	0.637	0.592	0.93	
			" 31	0.640	0.605	0.94	
7	26	"	Aug. 18	0.605	0.583	0.96	
8	64	"	" 21	0.631	0.591	0.94	
9	29	"	" 22	0.680	0.643	0.94	
			Sept. 6	0.043	0.594	0.92	
10	39	"	" 6	0.608	0.584	0.95	
11	67	"	" 14	0.637	0.577	0.91	
12	24	"	" 14	0.631	0.576	0.91	
13	19	"	" 20	0.653	0.624	0.955	
14	17	Female.	" 19	0.608	0.562	0.925	
15	32	"	" 21	0.617	0.569	0.92	
16	40	"	" 21	0.628	0.590	0.95	
17	32	"	" 22	0.645	0.612	0.95	
<b>Maximum.....</b>				0.680	0.643	0.98	47.5
<b>Minimum.....</b>				0.605	0.542	0.86	45.8
<b>Average.....</b>				0.634	0.593	0.985	45.2

*Pulmonary Infections.*

*Pneumonia Cases.*—We had the opportunity to study only two such cases, one of lobar pneumonia and one of bronchopneumonia, both of which are given in Table II.

In the first case (lobar pneumonia) the first venous puncture was made 2 days before the crisis and the results show that both titrated chloride and ionometric value were below the normal; the very low chloride content, however, seems in part to have been compensated by an increase in the other electrolytes, so that the ionometer equivalent was only just under the lower normal limit. Blood taken immediately after the crisis showed that both values had risen just above the lower normal limit, the ratio between them being approximately that normally found; the same was found to be true in the blood taken a few days later.

The second case (bronchopneumonia) did not in any of the three specimens of blood differ significantly from the normal.

*Cases of Pulmonary Tuberculosis.*—Since cases of pneumonia at the time were rare we studied another pulmonary infection, examining the blood and plasma in nineteen specimens of blood obtained from fifteen cases of pulmonary tuberculosis.

It appears from Table II that there was a very striking agreement between chloride concentration and ionometric chloride equivalent. The ratios between these varied within substantially the same limits as in normal plasmas (compare Table I).

A frank decrease in both values was found in five cases of tuberculosis having ionometric NaCl equivalents below 0.625 per cent and sodium chloride percentages below 0.580. Two cases showed ionometric NaCl equivalents slightly below the normal limit, i.e. 0.629 per cent, these cases having, respectively, a sodium chloride content of 0.589 and 0.596; i.e., close to the normal lower limit.<sup>3</sup>

The question arises as to whether there was anything in the condition of these patients which might explain the decrease of electrolytes in the plasma.

<sup>3</sup> In consideration of the small number of normal bloods examined, we shall consider only an ionometric NaCl equivalent below 0.630 and a NaCl concentration below 0.590 per cent as abnormally low.

TABLE III.  
*Diseases of the Kidneys.*

No.	Age.	Sex.	Date.	N <sub>o</sub> Cl equivalent of plasma.	N <sub>o</sub> Cl equivalent of plasma (extra- cellular fluid).	N <sub>o</sub> Cl content of plasma (extra- cellular fluid).	N <sub>o</sub> Cl equivalent of plasma.	Cell volume.	Diagnoses.	Albuminuria (systolic).	Urinary specific gravity to 6° C. (Strasen- berg test).	Non-protein ni- trogen of blood.	Edema.
1	60	Female.	May 10	0.6280.563	0.95	0.33	38.0	per cent	Nephrosclerosis.	Traces.	Normal.	0	
2	58	Male.	" 3	0.6300.589	0.89	X		per cent	Uremia.	About 6.	Increased.	0	
3	17	Female.	" 11	0.6330.632	0.97	0.31	42.1	per cent	Nephritis, second stage.	Good.	Normal.	0	
4	21	"	" 23	0.6580.613	0.93	0.29	40.5	per cent	Nephritis and nephrosis.	215	Good. Fixation.	"	+
5	20	Male.	" 4	0.6310.579	0.92	0.27	45.0	per cent	Renal tubercu- losis and amy- loid degenera- tion.	150	" 10.	"	+
6	25	"	" 13	0.6310.590	0.93	0.31	46.8	per cent	Nephritis and nephrosis.	130	" 10.	"	++
			Aug. 19	0.6310.612	0.97	0.35	40.0	per cent	Uremia.	20.	Fixation.	Greatly in- creased.	+

The mere presence of the tuberculous infection in itself can hardly be considered the cause since in that case the decrease should have been more frequent as ten of the patients were in the third stage of pulmonary tuberculosis. Neither hemoptysis, prevalence of bacteria in the sputum, pneumothorax, nor pleurisy could be correlated with this decrease in the electrolytes. In four out of the five patients with marked decrease in electrolytes the pulmonary process was very wide-spread, these being the

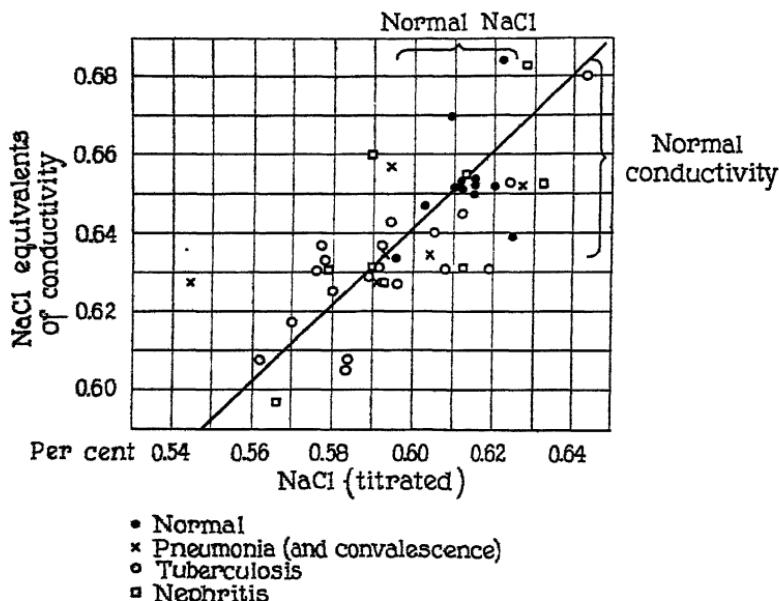


FIG. 2. Relation between NaCl titrated and NaCl equivalents of conductivity.

only patients that were considered to have a very bad prognosis. On the other hand, the fifth of these patients was convalescent and had at two previous examinations failed to show any decrease in the serum electrolytes.

#### *Diseases of the Kidneys.*

Ten specimens of blood from six patients were examined. All the cases were of a severe nature. The classification and symp-

toms are also given in Table III. This group of patients is, of course, a heterogeneous one and we only claim to have made a preliminary survey as to the relation between variations in chloride and conductivity of the plasma in these conditions. In only one case—the last—was there a marked decrease of ionometer and chloride values. In all the other cases the values were normal or nearly so. The agreement between ionometer and chloride variations was very good, most of the ratios falling within the normal ranges. A decrease of plasma chlorides was accompanied by a low conductivity.

Fig. 2 shows the relation between the NaCl equivalents of the conductivity and titrated NaCl in all the cases examined.

#### DISCUSSION AND CONCLUSIONS.

1. The ionometer of Christiansen offers a simple and quick method of measuring conductivity. For expressing the results obtained with this apparatus the term NaCl equivalent of the conductivity is introduced; *i.e.*, the concentration of sodium chloride in water required to give the same conductivity when measured with the ionometer. Conductivity measurements on plasma with the ionometer have been found to give an approximate indication of the chloride fluctuations in the plasma and may be employed to indicate whether marked changes in chloride concentration have occurred.
2. The chloride concentration of twelve normal plasmas, titrated and expressed as NaCl, varied between the limits 0.625 and 0.595 per cent with an average of 0.613 per cent.
3. The sodium chloride equivalents of the conductivity of the same normal plasmas when measured by the ionometer varied between 0.684 and 0.634 with an average of 0.653 per cent.  
The ratio  $\frac{\text{NaCl equivalent of conductivity}}{\text{Titrated NaCl per cent}}$  varied between 0.978 and 0.907 with an average of 0.939 per cent.
4. The parallelism of the change on the chloride concentration and in the conductivity under certain pathological conditions suggests that the fall in conductivity is due under these conditions to decrease in electrolytes rather than to increase in protein content.

5. The figures for corpuscle chlorides given in the tables indicate that the fall in plasma chloride was not caused by a chloride shift from plasma to corpuscles due to increase in CO<sub>2</sub> tension (Fridericia (7) and others).

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## A NOTE ON THE KRAMER-TISDALL METHOD FOR THE DETERMINATION OF CALCIUM IN SMALL AMOUNTS OF SERUM.

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In 1921 Kramer and the writer<sup>1</sup> described a simple method for the determination of calcium in serum. During the past year and a half several hundred determinations of the calcium content of serum have been made in this laboratory using a slight modification of the above method. The modification has further simplified and shortened the procedure, while the results obtained are identical with those secured by the use of the original technique.

The method as originally described consists in the precipitation of the calcium as calcium oxalate in a centrifuge tube. The precipitate was then driven to the bottom of the tube by the use of the centrifuge and subsequently washed three times with 2 per cent ammonia. The supernatant fluid was removed each time by the use of a specially constructed siphon. The modification consists in a change in the technique used to wash the precipitate. The original procedure as modified is as follows.

2 cc. of fresh serum are measured into a 15 cc. graduated centrifuge tube containing 2 cc. of water. *The outside diameter of the tube should be 6 to 7 mm. at the 0.1 cc. mark.* 1 cc. of a saturated solution of ammonium oxalate is added. The contents of the tube are then thoroughly mixed by holding the tube at the upper end and tapping the lower end with the finger giving it a circular motion. The mixture is allowed to stand for half an hour and the contents are again mixed. The tube is then

<sup>1</sup>Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, **xlvii**, 475.

centrifuged at about 1,500 revolutions per minute for 5 minutes. The supernatant fluid is removed by simply inverting the tube for a moment and allowing the fluid to flow out. The precipitate generally is not disturbed at all, but if it should be disturbed the tube can readily be returned to its original position before any escapes. The mouth of the tube is wiped with a cloth, and 2 per cent ammonia (2 cc. of concentrated ammonia to 98 cc. of water) is added up to 4 cc. This should be done so that it washes down the sides of the tube. The fluid is thoroughly mixed by tapping the tube with the circular motion until a slight amount of the precipitate spirals up the center of the fluid. All the precipitate does not need to be mixed with the fluid. The tube is centrifuged for 5 minutes and the supernatant fluid again removed by decantation. This constitutes one washing. The procedure is repeated making two washings in all. The crystals are then dissolved by the addition of 2 cc. of approximately N sulfuric acid. The tube and contents are heated in a boiling water bath for about 1 minute and titrated with 0.01 N potassium permanganate to a definite pink color which persists for at least 1 minute. The permanganate is delivered from a micro burette graduated in 0.02 cc. The number of cubic centimeters of 0.01 permanganate used multiplied by ten equals the number of milligrams of calcium per 100 cc. of serum.

It is seen that two washings have been substituted for three and the supernatant fluid removed by simple decantation instead of by the use of a specially constructed syphon. The amount of fluid left after decanting is generally 0.1 cc., while after the use of the syphon 0.3 cc. is left. By calculation it is found that after two washings by decantation 0.0005 mg. of ammonium oxalate is left to interfere with the titration of the calcium oxalate and after three washings with the use of the syphon 0.001 mg. of ammonium oxalate is left. In both instances these amounts are negligible as they constitute an error of less than  $\frac{1}{10}$  of 1 per cent with the average amount of calcium present in serum. A comparison of the results obtained by the original technique and the modified technique with solutions containing known amounts of calcium is given in Table I.

TABLE I.

Amount of Ca present. <i>mg.</i>	Amount of Ca found by original technique. <i>mg.</i>	Error. <i>per cent</i>	Amount of Ca found by modified technique. <i>mg.</i>	Error. <i>per cent</i>
0.2	0.200	$\pm 0.0$	0.196	-2.0
0.2	0.196	-2.0	0.198	-1.0
0.3	0.298	-0.6	0.300	$\pm 0.0$
0.4	0.394	-1.5	0.398	-0.5

Attention is directed to the length of time allowed for complete precipitation of the calcium. Clark<sup>2</sup> has made the following statement. "While the dilution of plasma with water (2 to 3 volumes) apparently allows a more rapid precipitation of the calcium, the reaction is not complete in the short time (30 minutes) allowed by Kramer and Tisdall." He adds, however, that this point was not completely proved. The results recorded in Table II show that the precipitation is completed in this short time.

TABLE II.

Serum No.	Diagnosis.	Time allowed for precipitation $= 30$ min.		Time allowed for precipitation $= 18$ hrs.	
		<i>mg. Ca per 100 cc. serum</i>	<i>mg. Ca per 100 cc. serum</i>	<i>mg. Ca per 100 cc. serum</i>	<i>mg. Ca per 100 cc. serum</i>
137	Bone tuberculosis.	10.1		9.9	
139	" "	10.5		10.5	
140	" "	9.2		9.2	
142	" "	9.5		9.5	

<sup>2</sup>Clark, G. W., *J. Biol. Chem.*, 1921, xlix, 487.



## THE FUNCTION OF THE PARATHYROIDS.\*

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Though the parathyroids have been known for more than 40 years, their function is still unknown. In 1909 MacCallum and Voegtlin (1) formed the theory that the parathyroids control the calcium metabolism and by doing so exert an influence on the nervous system. This theory was based upon two important findings in parathyroidectomized dogs; namely (a) the beneficent action of calcium salts on the symptoms, and (b) the decrease in the calcium content of blood and tissues. They could also demonstrate an increased excretion of calcium in the first days following the operation and they maintained that all the symptoms were due to calcium deficiency. Voegtlin and MacCallum (2) later abandoned this view and returned to the previous theory that a poison is the cause of the symptoms following parathyroidectomy.

The theory was abandoned by its authors chiefly because a rather concentrated solution of sodium chloride in large doses acted on the symptoms in the same way as calcium salts (3), contrary to what should be expected according to the experiments of Loeb (4), and because calcium salts, though checking the nervous symptoms for some time, failed to prolong the life of the parathyroidectomized animals.

The first obstacle against accepting the theory is probably removed by the recent work of Loeb (5) who showed that all

\* These studies were aided by a grant from the Wright fund and the Freia Chocolate Factory's fund for medical research of the University of Christiania. A preliminary report was published in the Proceedings of the Society for Experimental Biology and Medicine (Salvesen, H. A.; *Proc. Soc. Exp. Biol. and Med.*, 1922-23, xx, 204).

salts act in the same way in regard to permeability, but in very different concentrations. He found, for instance, that a m/8 solution of NaCl acts like a m/1,000 solution of CaCl<sub>2</sub>. What is true for permeability may also be true for irritability; in fact it was proved to be so by Joseph and Meltzer (3).

Concerning the objection that calcium salts fail to preserve the life of completely parathyroidectomized animals, it must be remembered that it is not known what is the fate of calcium injected into these animals; it may be eliminated again very rapidly. That calcium salts orally administered fail to preserve life may be due to bad absorption; it has never been shown that this calcium is absorbed at all. There are, moreover, many reports in the literature that calcium salts have preserved completely parathyroidectomized dogs; the first report was made by Frouin in 1909 (6), a paper which seems to have been forgotten. The opponents of the calcium theory have always maintained that accessory glands have come into action and saved these dogs. The latest report is made by Luckhardt and Goldberg (7), who claim to have preserved the life of completely parathyroidectomized dogs by oral administration of calcium lactate.

In experiments which have been undertaken in this laboratory for 2½ years, we have succeeded in preserving completely parathyroidectomized dogs by calcium administration for almost 2 years after the operation. It is the intention of this paper to show that calcium deficiency is the cause of the symptoms of parathyroidectomy and that the theory of MacCallum and Voegtlín (1) is probably right. This is shown by: (a) the chemical findings of the blood in parathyroid insufficiency; (b) experiments to determine the fate of calcium salts injected in tetanic dogs; and (c) experiments on completely parathyroidectomized dogs preserved by calcium treatment. The chemical methods used in this work were the following: the blood sugar was determined by the method of Hagedorn and Jensen (8); the alkali reserve by the method of Van Slyke and Cullen (9); serum calcium by the method of Kramer and Howland (10); and inorganic phosphorus by the method of Tisdall (11).

1. *The Blood Chemistry of Parathyroid Insufficiency.*—The observation of MacCallum and Voegtlín (1) that blood calcium drops following parathyroidectomy was confirmed by Hastings

and Murray (12) who found that when the serum calcium was below 7 mg. per 100 cc. tetany occurred. Our own findings confirmed this. In seven partially parathyroidectomized dogs tetany never occurred, and blood calcium never dropped below 7 mg., but was usually about 8 mg. and in a few weeks was again restored to the normal value of 10 mg. All the completely parathyroidectomized dogs developed violent tetany (ten dogs), their serum calcium dropped and was, when tetany occurred, always below 7 mg. There seemed to be a certain relation between the degree of the lowering of calcium and the violence of the symptoms. The inorganic phosphorus of the blood serum increased considerably; after the initial rise the phosphorus might drop again, but still seemed to be decidedly above the normal

TABLE I.  
*Dog 12. Complete Parathyroidectomy Aug. 21, 1922.*

Date.	Weight.	Blood sugar.	Alkali reserve.	In 100 cc. serum.		Remarks.
				Ca	Inor- ganic P.	
1922	kg.	per cent	vol. per cent	mg.	mg.	
Aug. 19	17.00	0.087	63.4	10.00	3.0	Operation Aug. 21.
" 23		0.087	47.2	3.50	12.5	Violent tetany. Calcium chloride intravenously.
" 24	15.95	0.098	53.4	5.68	6.3	No tetany. Calcium chloride administration.
" 26	15.65	0.104	63.7	6.54	6.5	No tetany.
" 27				5.16	6.3	Mild tetany.

value. This confirms Greenwald's (13) pioneer work of 1911 and 1913. The changes illustrated by Table I show the findings in one of the dogs.

*2. The Fate of Intravenously Injected Calcium Chloride in Parathyroidectomized Dogs.*—There are many reports in the literature of immediate relief of all the symptoms following injection of calcium salts in tetanic dogs. But the effect of the injection usually has been very short and the animals have died in spite of the treatment. In the present experiments, three of the completely parathyroidectomized dogs were treated with intravenous injections of a 10 per cent solution of calcium chloride. A total of 2 gm. a day usually was injected. This checked the

symptoms, the dogs were completely normal for a while, but almost invariably there was violent tetany again the next day which was checked by a new injection, and this was repeated day after day. It is easy to calculate that such doses of calcium chloride must raise the calcium content of the body fluids considerably. But whenever convulsions reappear after the injection, the serum calcium always was found to be down to the same low level again, which shows that the injected calcium is disposed of in some manner.

To find what happens to the injected calcium, three of the tetanic dogs were given a fixed dose of calcium chloride intravenously, and the urine and feces for 24 hours were analyzed for

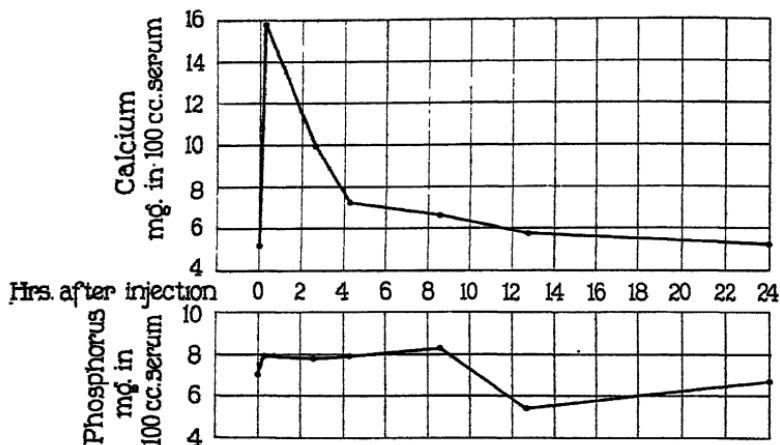


FIG. 1. Calcium and phosphorus in serum following the injection of 1.6 gm. of calcium chloride intravenously in Dog 11, weight 14.5 kilos.

calcium by the methods of McCrudden (14). The previous day and the day of the experiment the dogs were given a calcium-free diet. The colon was rinsed out at the beginning and close of the period. The curves for the serum calcium and phosphorus were determined during this period.

Fig. 1 shows the effect of the injection of 1.6 gm. of  $\text{CaCl}_2$  on the serum calcium and phosphorus in one of the dogs. It is seen that 15 minutes after the injection the calcium was 15.6 mg. and decreased rapidly. 24 hours after, the calcium had the same

value as before the injection; that is, there had disappeared from the blood an amount of calcium exactly corresponding to the amount injected. There was also a rise in the phosphates. The curves for the other dogs closely resembled this curve. As Table II shows there was excreted an amount of calcium corresponding to the amount injected, but more than nine-tenths were excreted in the feces and less than one-tenth in the urine.

This rapid disappearance of the injected calcium may explain why the calcium treatment reported by previous workers failed to keep the animals alive. Only a few hours after the injection of a relatively large dose of calcium chloride the blood calcium is down to a level where tetany usually occurs. The experiments also show that in parathyroidectomized dogs there is a lowered threshold for the excretion of calcium through the intestines.

TABLE II.

*Dog 11. Calcium Excretion in Urine and Feces. June 20 to 21, 1922.*

Urine Ca.	Feces Ca.	Total.
gm.	gm.	gm.
0.042 Injected. ...	0.504	0.546
		0.578

*3. Experiments on Completely Parathyroidectomized Dogs Preserved by Treatment.*—Of ten dogs which were completely parathyroidectomized (enough thyroid tissue was left to prevent cachexia), five died untreated within 3½ days; one died 22 days after (the condition of this one was complicated by extensive necrosis of three legs following unsuccessful intravenous injection of calcium chloride); and two other dogs, also treated with calcium, were preserved. They were given 1 gm. of calcium chloride intravenously twice a day, but after a week or two the doses employed could be lowered and at last omitted. During this time the dogs were fed a milk diet. Two other dogs recovered spontaneously during milk feeding by stomach tube; that this contributed to successful preservation will be seen from the following experiments. These four dogs could be kept alive indefinitely on a milk diet; one lived for 21 months and was then killed; the others were either killed or, at will, brought into tetany from which they died. These dogs, which appeared

absolutely normal could, whenever it was wanted, be brought into tetany, usually within 24 hours, by changing the diet to meat. It is obvious that this condition formed an excellent opportunity for studying the various factors involved in the production of tetany.

TABLE III.  
Dog 2. Complete Parathyroidectomy Mar. 14, 1921.

Date.	Weight.	Blood sugar.	Alkali reserve.	Ca in 100 cc. serum.	Remarks.
1921	kg.	per cent	vol. per cent	mg.	
Apr. 6	10.73	0.099	52.5	5.22	Recovered after violent tetany, but still sick.
" 26	10.75	0.098	54.3	5.87	Absolutely normal.

Though to all appearances normal, these dogs showed one characteristic finding in the blood: *the calcium was still low*. Table III shows an example of this. The calcium varied to some extent, but was usually between 5 and 6 mg. per 100 cc. of serum.

TABLE IV.  
Dog 2. The Effect of Meat Diet on the Latent Tetanic Condition 13 Months after Complete Parathyroidectomy.

Date.	Weight.	Blood sugar.	Alkali reserve.	Ca per 100 cc. serum.	Remarks.
1922	kg.	per cent	per cent	mg.	
Apr. 26	11.45	0.091	55.3	6.66	No symptoms. To all appearances normal. Meat diet started.
" 27		0.095	57.7	5.70	No symptoms.
" 28					
11.00 a.m.	11.45	0.099	47.6	5.28	Mild tetany.
7.30 p.m.				4.96	Violent tetany. Injection of calcium chloride.
Apr. 29	11.45			5.56	No symptoms.

The inorganic phosphorus was usually at the upper border of the normal level or above. This low calcium is very significant and evidently characterizes *latent* tetany in contrast to *cured* tetany.

(a) *The Diet in Latent Tetany.*—On a milk diet (minimum 500 cc. a day with bread or porridge) these dogs after the first critical period was over could be kept without symptoms for as long a time as desired. Meat always produced tetany (see Table IV), loss of appetite, and depression; prolonged meat feeding killed two of the dogs. It is an old experience that milk is beneficial and meat harmful to parathyroidectomized dogs. The reason for the difference will be seen from the following experiments.

(b) *Why Milk Prevents Tetany.*—Experiments with various forms of diet showed that it was the withdrawal of milk which produced tetany, not the meat diet in itself, and milk, therefore,

TABLE V.

*Dog 2. The Effect of Milk, Poor in Calcium, 16 Months after Complete Parathyroidectomy.*

Date.	Weight.	In 100 cc. serum.		Ca in 500 cc. milk.	Remarks.
		Ca	Inor- ganic P.		
1932		mg.	mg.	gm.	
Sept. 6	11.10	6.20	6.1	0.118	500 cc. calcium-poor milk + porridge.
" 7				0.039	No symptoms. Quiet, no appetite. Food has to be given by stomach tube.
" 8	3.30	5.5			Fully developed tetany.

must contain a substance which prevents tetany. This substance proved to be calcium, of which the milk used contained 1.2 gm. per liter, corresponding to about 10 gm. of calcium lactate. It was discovered, that when the amount of sodium oxalate calculated to precipitate all this calcium was added to the milk, at least 90 per cent was carried down. Less than this was used in the experiments in order not to get an excess of oxalate in the milk, the precipitate was separated from the milk by centrifugation, and this calcium-poor milk, which always was analyzed for calcium before feeding, was now useless in preventing tetany. Usually within 24 hours the dogs developed violent tetany when fed this milk. Table V illustrates one of the experiments.

(c) *Calcium Administration.*—On the other hand, if an amount of soluble calcium salt was given corresponding to the Ca in the

amount of milk, which prevents tetany, the dogs remained normal on any diet; now when meat was given in excess, the dogs increased in weight and were normal in all their actions. The reason why meat produces tetany, therefore, must be due to the fact that it is very poor in calcium (see Table VI).

TABLE VI.

*Dog 2. The Effect of Meat Given with Calcium Lactate and Meat Alone,  
18½ Months after Complete Parathyroidectomy.*

Date.	Weight	In 100 cc. serum.		Remarks.
		Ca	Inor-ganic P.	
1922 Aug. 26	kg. 11.15	mg. 5.00	mg. 6.4	No symptoms. 350 gm. minced horse meat + 50 gm. fat + 4.6 gm. calcium lactate in 200 cc. water daily.
Oct. 2 " 3 9.45 a.m.	10.40 3 •	5.12 3.66 5.26	6.0 5.4 6.3	No calcium. Meat and fat as before. Violent tetany. At 10 a.m. given 4.6 gm. calcium lactate in 200 cc. water. Normal. Eats meat and fat with usual appetite.

TABLE VII.

*Dog 12. The Effect of a Single Dose of Calcium Lactate upon Calcium and Inorganic Phosphorus of Blood Serum and the Clinical Symptoms.*

Date.	Weight	In 100 cc. serum.		Remarks.
		Ca	Inor-ganic P.	
1922 Oct. 6 10.15 a.m.	kg. 15.50	mg. 4.39	mg. 6.9	Violent tetany. At 10.45 a.m. given 9.2 gm. calcium lactate in 200 cc. water.
2.30 p.m.	•	6.04	5.4	No symptoms.

It was possible by giving large amounts of calcium lactate by stomach tube to restore the calcium content of the blood almost to the normal level. From this condition it took a longer time than usual to produce tetany on a milk-free diet, and when tetany occurred the serum calcium was always found lowered again.

If tetany was produced from the latent stage a single dose of calcium lactate (5 to 10 gm.) usually checked all the symptoms. Analysis of serum calcium then always showed that at the moment

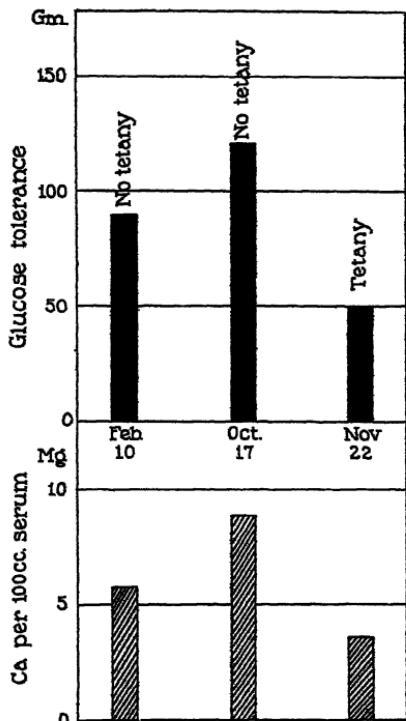


FIG. 2.

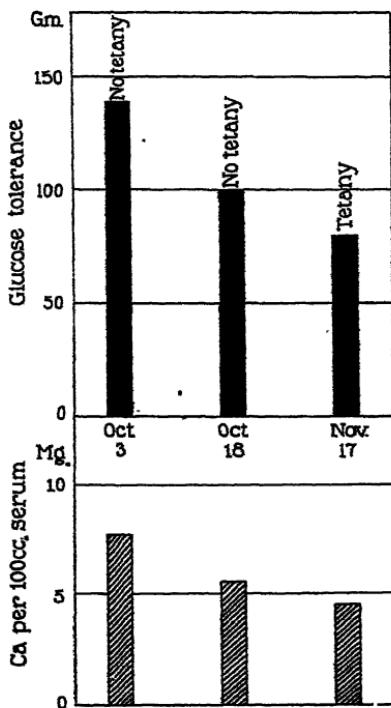


FIG. 3.

FIG. 2. Relation between serum calcium and glucose tolerance in Dog 2, weight 11.6 kilos. The upper columns represent the glucose tolerance; that is, the smallest amount of glucose given orally which produces glycosuria. On Nov. 22, however, the glucose tolerance actually was lower than shown in the figure, as the urine after the administration of 50 gm. of sugar contained 10 per cent of glucose, and there were excreted 6.2 gm. of glucose in the urine.

FIG. 3. Relation between serum calcium and glucose tolerance in Dog 12, weight 15.5 kilos. The tolerance on Oct. 3 actually was higher than indicated by the figure, as the amount of glucose given did not produce glycosuria, and on Nov. 17 the tolerance was lower, as the urine after the administration of 80 gm. of sugar contained 8 per cent glucose, and there were excreted 4 gm. of glucose in the urine.

when the symptoms were relieved there was an increase in the calcium content of the blood, showing that it is the actual absorption of calcium which cures the symptoms. Table VII illustrates this. The same increase in serum calcium was seen when milk checked the symptoms.

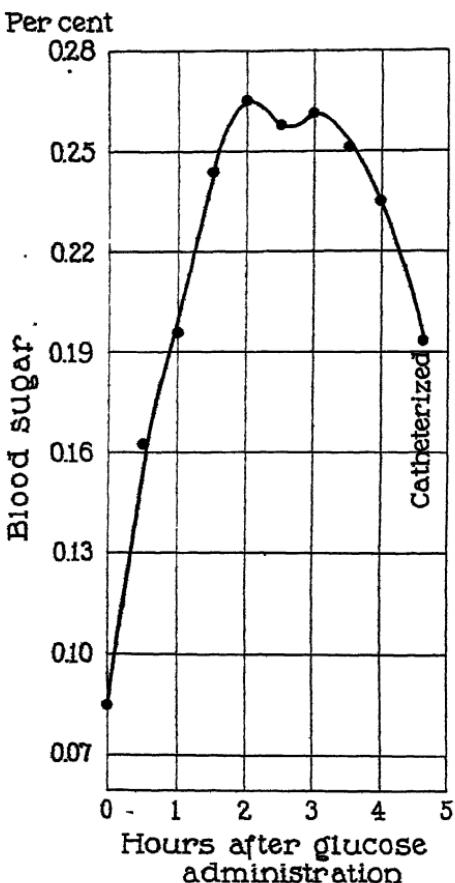


Fig. 4. Dog 2, weight 11.6 kilos. Blood sugar curve during glucose tolerance test. Oct. 17, 1922, 10.40 a.m., 120 gm. of glucose in 20 per cent solution were given by stomach tube. At 3.30 p.m. catheterized; urine contained 0.36 gm. of glucose.

The fact that the blood calcium remained at a low level in these dogs might be interpreted as indicating that it is not the lowered calcium which is the cause of the symptoms; but the experiments in all four dogs showed that whenever the animals were brought into tetany from a symptom-free condition, the calcium was always *still further lowered*; and whenever the animals by administration of calcium or milk were made normal again, the blood calcium was *always higher* than during the symptoms of insufficiency. There seems to be an "adaptation"

TABLE VIII.

*The Effect of Glucose Feeding on the Inorganic Phosphorus Content of Serum.*

Dog No.	Date.	Weight. kg.	Per 100 cc. serum.		Blood sugar. per cent	Remarks.
			Ca mg.	Inorg- anic P. mg.		
12	Nov. 17 10.00 a.m.	13.50	4.7	6.9	0.099	Tetany. At 10.30 a.m. given 80 gm. glucose in 20 per cent solution.
					4.1	12.45 p.m. catheterized, urine contains 4 gm. glucose.
2	Nov. 22 11.00 a.m. 2.45 p.m.	9.95	2.52	5.4	0.111	Tetany. At 11.15 a.m. given 50 gm. glucose in 20 per cent solution.
				4.9	0.227	3.00 p.m. catheterized, urine contains 6.4 gm. glucose.

of some sort to the lowered calcium, but a further decrease promptly causes the characteristic symptoms. The inorganic phosphorus in these latent tetanic dogs showed no regularity except a certain tendency to keep at a high level, otherwise there was no relation between the phosphorus content of the blood and the clinical symptoms.

(d) *Experiments on the Carbohydrate Metabolism.*—Experiments made on two of these dogs showed that the tolerance for glucose (in 20 per cent solution by stomach tube) was distinctly lowered during the latent stage. When the dogs were brought into

tetany, the tolerance went down to very low values. On the other hand, when the blood calcium was increased by forced calcium feeding, the tolerance was markedly increased, and in one of the dogs it was impossible to induce glycosuria under these circumstances. When glycosuria developed the blood sugar curves went far above the threshold and they indicate that the lowered tolerance is not due to increased permeability of the kidneys but to a functional disturbance of the glycogen-forming organs. Figs. 2, 3, and 4 illustrate these experiments.

It was noticed that the inorganic phosphorus of serum might drop as a result of the glucose feeding (Table VIII). This observation may support the theory of Embden and coworkers (15), that phosphoric acid plays an important rôle in the metabolism of sugar.

#### DISCUSSION.

From these experiments it is evident that the characteristic feature in the chemistry of parathyroid insufficiency is the drop in blood calcium which is the more marked the more parathyroid tissue there is removed. To this probably must be added the increase in the phosphates; and the quantitative experiments of Binger (16) indicate that there is a relation between the rise in phosphates and drop in calcium. But it is the drop in calcium which is the cause of the symptoms. This is shown by the following facts: all the symptoms are relieved and the dogs made absolutely normal for a while by intravenous injections of calcium chloride; the symptoms reappear after a certain time, but not until the blood calcium again has reached the same low level as before. The injected calcium is very rapidly excreted through the intestines and only small amounts appear in the urine; even large doses disappear in a remarkably short time. This accounts for the fact that it has been so difficult to preserve parathyroidectomized animals by calcium injection.

It has further been shown that completely parathyroidectomized dogs *can* be preserved by calcium treatment, and this is a still further proof that the symptoms are due to the calcium decrease. In these dogs, which are in a state of latent tetany characterized by a low and very unstable blood calcium, and which require a milk diet rich in calcium to keep symptom-free, tetany can always

be produced by calcium withdrawal from the food. This is followed by a further drop in the blood calcium, and the dogs can always be saved by calcium administration. The relief of the symptoms is always accompanied by an increase in blood calcium. The hitherto unexplained fact that milk is beneficial to parathyroidectomized animals, and, as has been shown in the present experiments, can protect latent tetanic dogs against tetany, is found to be due to the calcium content of the milk which makes it equal to a 1 per cent solution of calcium lactate.

The glucose tolerance experiments show that the supposed influence of the parathyroids on the sugar metabolism is only an indirect one; the sugar tolerance sinks because the blood and body fluids are poor in calcium. When the blood calcium is restored almost to the normal level the tolerance for sugar is practically normal. That this symptom is due also to a lack of calcium further supports the calcium deficiency theory.

It is apparent from the experiments that in parathyroidectomized dogs there is a lowered threshold for the excretion of calcium in the intestines, and this is apparently the cause of the calcium deficiency and thereby of all the symptoms. It still remains to be explained how the parathyroid hormone prevents this unlimited excretion of calcium through the intestines. The behavior of the blood calcium indicates that the actual recovery of completely parathyroidectomized dogs is not due to compensatory hypertrophy of accessory glands, as the action of these would be to restore the blood calcium to the normal level. The "adaptation" to a low calcium level, which in the beginning causes tetany, is not explained.

It will follow from these results that if a disease is due to parathyroid insufficiency it will show a low blood calcium.

How lactose acts in preventing tetany as shown by Dragstedt (17) remains to be learned; determinations of blood calcium in these dogs preserved by Dragstedt's method may give some information.

#### CONCLUSION.

The symptoms of parathyroid insufficiency are due to calcium deficiency. The parathyroids control the calcium level of the blood and by doing so they influence the function not only of the muscle and nerve tissues, but probably of all the organs.

The successful outcome of the operations is due to the valuable help of Professor Dr. S. Torup to whom the author wishes to express his thanks.

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## CONSTITUENTS OF THE WAX-LIKE COATING ON THE SURFACE OF THE APPLE.

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On consulting the literature it was found that very little has hitherto been recorded respecting the constituents of the wax-like coating on the surface of the apple.

In 1895 Seifert (1) noted the presence, in a chloroform extract of the dry peels, of a wax-like substance melting at  $64^{\circ}$ , and another compound of higher melting point,  $234^{\circ}$ , which seemed to resemble a compound called vitin, previously isolated from grape skins (2) by the same investigator. The compound resembling vitin was not obtained in a crystalline condition, and since no attempt was made to show that the low melting substance was pure, it is doubtful whether these preparations were homogeneous. Thomae (3) in a preliminary communication, 1911, without reference to Seifert's previous work, states that he obtained from an ether extract of apple parings, a crystalline product melting above  $200^{\circ}$ , which was easily soluble in hot alcohol, but which separated again on cooling. Further characterization or identification was not reported. In a second publication (4) the same author calls attention to the fact that the crystalline product mentioned in his first note was not homogeneous, and could be resolved into two substances, one of high, and the other of low melting point. The first compound was not examined. The second possessed wax-like properties. The two substances were separated by treatment with cold ether, when the low melting compound dissolved while the other one remained insoluble. The ether-soluble constituent was crystallized from absolute alcohol. It melted at  $68.5^{\circ}$ . The only published reference

to the occurrence of a paraffin hydrocarbon in apple peels is found in a very interesting paper by Power and Chesnut (5) on the odorous constituents of the apple. By subjecting fresh apple parings to distillation in a current of steam these investigators were able to isolate, in addition to the odorous constituents, a very small quantity (0.04 gm.) of a hydrocarbon which melted sharply at 63°. The crystalline substance, according to these investigators, appeared to be slightly impure triacontane,  $C_{30} H_{62}$ , which is supposed to melt at 65.5°. Finally, it may be mentioned that Molisch (6) noticed the presence of oily droplets on the fruit of *Malus coronaria*. No characterization or identification of this oily material was reported.

From this summary of the literature it is apparent that our knowledge of the nature of the chemical substances which form the wax-like coating of apples is extremely incomplete. For this reason, and because of the bearing that a knowledge of these substances may have on the solution of storage scald, the writer has undertaken an investigation of the non-volatile wax-like substances covering the epidermis of the apple.

#### EXPERIMENTAL.

The material employed in this investigation was obtained from the peels of two varieties of apples, Ben Davis and Black Ben Davis, which were grown on the experimental farm at Arlington, Virginia. The horticultural differences between these two varieties are so slight that no hesitation was felt about using the combined material in order to obtain a sufficient supply for the investigation.<sup>1</sup>

In paring the apples by a machine care was taken to exclude, as far as possible, any fleshy portion of the fruit. Rapid dehydration of the freshly cut peels was facilitated by spreading the material on trays and drying it in a current of warm air.

<sup>1</sup> The apples and facilities for paring the fruits were kindly furnished by Dr. J. S. Caldwell in charge of the fruit and vegetable utilization laboratory, Office of Horticultural and Pomological Investigations, Bureau of Plant Industry. To Dr. Caldwell and others of that laboratory who aided in many ways, the writer wishes to express his utmost appreciation. Thanks are also due Mr. Paul Williams, of this laboratory, for help in preparing the material and making many of the tedious extractions.

The dry peels were then coarsely ground and extracted in a large Soxhlet apparatus with U.S.P. ether. Frequent changes of solvent were necessary in order to eliminate danger of bumping caused by the separation of solid matter from the ether.

The light green residue obtained by the complete evaporation of the combined ether extracts became powdery in the dry state and was resinous to the touch.

In order to remove most of the colored impurities, without dissolving much of the other substances, it was necessary to shake the ether-soluble residue several times with cold 80 per cent acetone. The acetone extracts have not been examined. Preliminary treatment of the white residue insoluble in 80 per cent acetone indicated that a separation of compounds could be effected by continued extraction with petroleum ether. This separation could also have been made by extracting the original dry peels first with petroleum ether and then with ether.

To facilitate extraction with petroleum ether the white residue was mixed with plaster of Paris and made to a paste by the addition of water. When the mass had set it was coarsely ground and extracted in a Soxhlet apparatus with petroleum ether (B.P. 30-50°). After the substances soluble in petroleum ether had been completely removed, the extraction was continued with ether.

#### *The Petroleum Ether Extract.*

##### *Identification of Triacontane, $C_{30}H_{62}$ , and Heptacosanol, $C_{27}H_{56}O$ .*

The pale greenish petroleum ether extract was evaporated to dryness and the residue subjected to repeated extraction with successive small quantities of cold and finally with hot petroleum ether (B.P. 40-50°). Each fraction was evaporated and the residue crystallized from hot acetone or absolute alcohol. Fractions having similar melting points were combined and again fractionally extracted with successive quantities of petroleum ether. After several repetitions of the foregoing process relatively few fractions were finally obtained whose melting points indicated approximate purity. These fractions were as follows:

Fraction No.	Melting point.*
	°C.
1	63 (predominating).
2	70
3	70-71.5
4	75-76
5	75-77
6	77-78
7	79-79.5
8	80-81.5 (next in quantity).

\* All melting point determinations made in the course of this investigation were obtained by the use of Anschütz short stem thermometers, which were standardized by the United States Bureau of Standards. No stem corrections were made.

In view of the fact that Fraction 1 appeared to be closely similar to the paraffin hydrocarbon obtained by Power and Chesnut (5) little doubt was felt that the two substances were identical. Considerable difficulty was experienced, however, in purifying this substance. In spite of the fact that the melting point was quite sharp at 63°, and that repeated crystallizations from acetone, absolute alcohol, and ethyl acetate failed to effect any change, the results of a combustion ( $C = 84.1, H = 14.4$  instead of  $C = 85.2, H = 14.8$ ) indicated that the compound was still impure. Treatment with phthalic anhydride (7) to remove impurities of an alcoholic nature also failed to produce the desired purification. The substance was finally obtained pure by boiling with alcoholic potassium hydroxide for 3 or 4 hours under a reflux condenser, and then filtering the cooled liquid. The insoluble portion was washed with alcohol and dried, after which it was freed from potassium hydroxide by solution in ether. Evaporation of the ether yielded a residue which, when crystallized from absolute alcohol, separated as satiny crystals. The melting point of the compound obtained by this treatment was 63.5-64°. After melting, resolidification occurred at 63-62.5°, and the compound remelted at 63.5-64°. Combustion results of the anhydrous substance are as follows:

0.1124 gm.: 0.1464 gm.  $H_2O$  and 0.3513 gm.  $CO_2$ .

0.1247 " : 0.1667 " " 0.3892 " "

Found.<sup>2</sup> C 85.23, 85.11; H 14.60, 14.98.

$C_{16}H_{32}$  requires: C 85.20, H 14.79.

<sup>2</sup> Calculations of C and H results throughout this paper are based on H = 1.008.

The melting point of triacontane is given by Krafft (8) as 65.6°. There can be little doubt, therefore, that this substance is triacontane.

The remaining fractions, with the exception of No. 8, were obtained in quantities too small for further identification and will have to be examined when larger quantities of crude material are available.

Fraction 8, melting at 80–81.5° was recrystallized from absolute alcohol and boiled under a reflux condenser with alcoholic potassium hydroxide for 3 or 4 hours. The solvent was then evaporated, finally under diminished pressure, and the residue extracted with hot petroleum ether (B.P. 40–50°), which was subsequently removed by distillation. The residue was crystallized from absolute alcohol and dried in a vacuous desiccator over phosphorus pentoxide. The pure compound melted at 81.5–82°, resolidified at 77.5–77°, and remelted at 81–81.5°. Treatment with alcoholic potassium hydroxide produced no apparent change. After being dried in a vacuous desiccator over phosphorus pentoxide, the substance was analyzed with the following results:

0.1100 gm.: 0.1368 gm. H<sub>2</sub>O and 0.3296 gm. CO<sub>2</sub>.

0.1189 " : 0.1507 " " 0.3564 " "

Found. C 81.71, 81.74; H 13.94, 14.20.

(C<sub>18</sub>H<sub>37</sub>)<sub>2</sub>CHOH requires: C 81.72, H 14.23.

It is evident from these results that this substance is identical with dimyristylcarbinol (9) or heptacosanol, C<sub>27</sub>H<sub>56</sub>O, whose melting point is given as 80.5–81.5°. This compound has been prepared by Kipping (9) from the ketone, myristone, (C<sub>18</sub>H<sub>37</sub>)<sub>2</sub>CO, by reduction with sodium and alcohol, but no natural source had hitherto been known.

In order further to confirm the identity of this substance with synthetic heptacosanol, the acetate was prepared by heating the dry compound with an excess of acetic anhydride for 3 hours. The product was then poured into a large volume of water and stirred until crystallization occurred, after which it was filtered, washed, and dried. One crystallization from 95 per cent alcohol yielded a product melting at 44–46°, and this melting point remained unchanged by a second crystallization. The acetate separated as colorless, glistening, rectangular plates melting at

44–46°, and remelting at 43–45°. The melting point of pure dimyristylcarbiny acetate,  $(C_{13}H_{27})_2CHO(CO \cdot CH_3)$ , is reported as 45–45.5° (9).

*The Ether Extract.*

*Isolation of a New Crystalline Alcohol, Malol,  $C_{30}H_{48}O_3$ .*

After removing colored impurities and constituents soluble in petroleum ether from the original extract of dried peels, the residue in the plaster of Paris was thoroughly extracted with ether in a Soxhlet apparatus. After evaporation of the solvent there was obtained a yellowish white powder. The entire quantity of this amorphous powder was dissolved in boiling 95 per cent alcohol and filtered by means of a hot water funnel when a small quantity of dark impurity remained on the filter. A voluminous precipitate formed in the filtrate on cooling. This was collected on a Buchner funnel, washed, and without drying, heated a short time with dilute alcoholic sodium hydroxide which caused the separation of a dark impure material. After filtration the solvent was removed by distillation under diminished pressure and the residue extracted with petroleum ether until impurities were no longer removed. The residue was finally dissolved in 95 per cent alcohol containing a small quantity of sodium hydroxide, the solution filtered, and hot water added to the filtrate to slight turbidity. Thereupon most of the alcohol was removed by evaporation when a copious precipitate of crystalline sodium salt separated from the liquid, and on cooling a further separation occurred. The sodium salt, after being washed with a little water, was recrystallized several times by solution in aqueous alcohol containing a slight excess of sodium hydroxide and evaporation of the greater portion of alcohol.

The mother liquors from these crystallizations yielded gelatinous precipitates with dilute hydrochloric acid which appeared to be different from any other product obtained, but nothing of a crystalline nature could be isolated.

The pure sodium salt obtained by the foregoing method was converted into the parent substance by solution in alcohol and addition of hot dilute hydrochloric acid. The precipitate, after thorough washing to eliminate sodium chloride, was obtained in

a pure state by several crystallizations of the dried material from absolute alcohol. When crystallized in this manner, the substance consisted of colorless, highly lustrous, prismatic needles. The first attempts to crystallize it from dilute alcohol resulted in the formation of amorphous precipitates which were difficult to filter. Crystallization was finally accomplished, however, by dissolving the amorphous material in boiling 95 per cent alcohol and adding hot water until a slight separation occurred. Removal of the flask from the source of heat and continual agitation after addition of several glass beads resulted in the formation of hair-like needles. There was some indication that the substance obtained by crystallization from dilute alcohol contained water of crystallization, most of which is gradually lost on exposure to the air.

Crystallized from absolute alcohol, the compound, when placed in the melting bath at room temperature and gradually heated, melts at 280-282°. If placed in the bath at 250° and heated fairly rapidly the melting point is raised to 284-285°. The anhydrous substance was obtained by drying in a desiccator over phosphorus pentoxide or by heating at 105° in a vacuum. The anhydrous compound had the following composition:

0.1337 gm.: 0.1260 gm. H<sub>2</sub>O and 0.3862 gm. CO<sub>2</sub>.

0.1225 " : 0.1160 " " 0.3536 " "

Found. C 78.77, 78.72; H 10.56, 10.61.

C<sub>30</sub>H<sub>48</sub>O<sub>3</sub> requires: C 78.88, H 10.60.

The sodium salt prepared according to the method already indicated, was extracted with ether to eliminate any parent substance, which is formed by hydrolysis during washing. After being dried at 125-130°, it was analyzed with the following results:

0.4326 gm.: 0.0626 gm. Na<sub>2</sub>SO<sub>4</sub>.

0.4869 " : 0.0699 " "

Found. Na 4.68, 4.64.

C<sub>30</sub>H<sub>47</sub>O<sub>3</sub>Na requires: Na 4.80.

These results indicate that the above mentioned substance possesses the formula C<sub>30</sub>H<sub>48</sub>O<sub>3</sub>. Since this compound does not appear to be identical with any other substance described in the literature, the designation *malol* is proposed.

*Malol*,  $C_{30}H_{48}O_3$ , is obtained from dilute alcohol in the form of fine hair-like needles and from absolute alcohol as large, highly lustrous, prismatic needles. From most of the other solvents in which it is soluble, or slightly so, it separates in an amorphous condition. Malol is practically insoluble in petroleum ether and water, sparingly soluble in ether, chloroform, ethyl acetate, acetone, cold ethyl alcohol, and glacial acetic acid. It is readily soluble in boiling 95 per cent or absolute ethyl alcohol from which it is easily crystallized. An alcoholic solution of malol is dextro-rotatory and exhibits muta-rotation. Malol shows the Liebermann-Salkowski cholesterol reaction. For example, if a small quantity of the substance is dissolved in acetic anhydride and a few drops of concentrated sulfuric acid are added, the solution assumes a pink color which slowly passes through violet to blue and finally to green.

*Diacetylmalol*,  $C_{30}H_{46}O_3(CO \cdot CH_3)_2$ .—Malol was completely acetylated by heating for several hours with an excess of acetic anhydride. After removal of the greater portion of acetic anhydride by distillation, the diacetyl derivative was slowly deposited from the liquid. This deposit was collected on a Buchner funnel by the use of suction, washed with cold 70 per cent alcohol, and dried. Acetyl derivatives are usually crystallized from 95 per cent alcohol, but in this case hydrolysis readily takes place when this solvent was employed. The dried product was, therefore, dissolved in petroleum ether (B.P. 30–50°) and obtained pure by partial evaporation of the solvent. Crystallized from petroleum ether, it separated in fine colorless needles. When placed in the melting bath at 180°, it melted at 199–200° with an evolution of gas, whereupon it resolidified and did not melt again under 300°. A chloroform solution of diacetylmalol is dextro-rotatory. Analysis of the compound gave the following results:

0.1287 gm.: 0.1115 gm. H<sub>2</sub>O and 0.3568 gm. CO<sub>2</sub>.

0.1059 " : 0.0909 " " 0.2927 " "

Found. C 75.60, 75.38; H 9.71, 9.62.

$C_{30}H_{46}O_3(CO \cdot CH_3)_2$  requires: C 75.49, H 9.69.

*Monoacetylmalol*,  $C_{30}H_{47}O_3(CO \cdot CH_3)$ .—In connection with a study of diacetyl derivatives of prunol (10) and oleanol (11) it was reported by the respective authors that when these deriva-

tives are dissolved in dilute alcohol and the solutions boiled for several hours, one acetyl group is eliminated. The same effect is produced on diacetylmalol when its solution in 70 per cent alcohol is boiled for 2 hours. The monoacetyl derivative obtained in this manner is deposited from the cooling solution in small colorless needles which melt indefinitely about 279–281° with apparent decomposition. The composition is as follows:

0.1136 gm. (anhydrous): 0.1031 gm. H<sub>2</sub>O and 0.3216 gm. CO<sub>2</sub>.

Found. C 77.20, H 10.17.

C<sub>30</sub>H<sub>47</sub>O<sub>8</sub>(CO·CH<sub>3</sub>) requires: C 77.10, H 10.04.

*Monomethylmalol*, C<sub>30</sub>H<sub>47</sub>O<sub>8</sub>(CH<sub>3</sub>).—A quantity of malol was dissolved in absolute alcohol and boiled with an excess of both sodium ethoxide and methyl iodide. After 3 hours, the excess of alcohol was removed by distillation and the product poured into water. The insoluble portion was later collected and dried. The substance was purified by solution in ether (shaking), washing with aqueous sodium hydroxide to remove unchanged malol, and finally with water. When air-dried or dried at 55°, the compound sinters at about 110° and melts at 170.5–171.5°. If placed in the bath at about 125°, it immediately melts and gradually resolidifies, and again melts at 170.5–171.5°. In the anhydrous condition it melts without previous sintering or melting, at 170.5–171.5°. The compound dried at 120–125° had the following composition:

0.1232 gm.: 0.1166 gm. H<sub>2</sub>O and 0.3576 gm. CO<sub>2</sub>.

Found. C 79.16, H 10.61.

C<sub>30</sub>H<sub>47</sub>O<sub>8</sub>(CH<sub>3</sub>) requires: C 79.08, H 10.71.

*Acetymethylmalol*, C<sub>30</sub>H<sub>46</sub>O<sub>8</sub>(CH<sub>3</sub>)(CO·CH<sub>3</sub>).—This substance was obtained by heating the monomethyl derivative for 1 hour with an excess of acetic anhydride. After evaporation of the greater portion of acetic anhydride, the concentrated liquid on cooling yielded a crystalline deposit which was purified by two crystallizations from dilute alcohol. Acetymethylmalol, dried at 125–130°, melted at 243–244°. Combustion results are as follows:

0.1218 gm.: 0.1109 gm. H<sub>2</sub>O and 0.3457 gm. CO<sub>2</sub>.

Found. C 77.40, H 10.21.

C<sub>30</sub>H<sub>48</sub>O<sub>3</sub>(CH<sub>3</sub>)(CO.CH<sub>3</sub>) requires: C 77.28, H 10.22.

#### DISCUSSION.

There are a number of substances described in the literature which possess the same formula and exhibit the same general chemical properties as malol.

Urson was first obtained from the leaves of *Arctostaphylos uva ursi* by Trommsdorff.<sup>3</sup> Gintl (12), who was the first to make a thorough study of urson, assigned to it the formula C<sub>30</sub>H<sub>48</sub>O<sub>3</sub>, gave its melting point as 263–266°, and described an acetyl derivative melting at 264°. Dodge (13), in 1918, also made a critical examination of urson and compared it with caryophyllin which appeared to be closely related. His preparation of urson (from *Arctostaphylos uva ursi*) melted at 285° and yielded a diacetyl and a monoacetyl derivative. The diacetyl derivative when heated lost acetic acid at 135° and melted at 165°. Nooyen (14) undertook an investigation of urson and its distribution in the plant kingdom and as a result of her work reported the melting point of urson as 273° and of its methyl ester as 148°. This investigator claims that urson contains no aldehyde, keto, methoxy, or hydroxy groups—a conclusion which is difficult to harmonize with the results of other investigators. Van Itallie (15) quite recently, 1921, isolated a compound from mistletoe (*Viscum album*) growing on the apple tree, which appeared to him to be urson. It melted at about 287°, but no further characterization was reported.

It is quite evident from the foregoing statements that the literature relating to urson is very contradictory and that further investigation of this product is needed.

Two other compounds possessing the formula C<sub>30</sub>H<sub>48</sub>O<sub>3</sub> are described in the literature. Caryophyllin, originally obtained from clove buds (16) and critically examined by Dodge (13), has a melting point of 310° and yields a diacetyl and a monoacetyl derivative, the latter melting at 260–265°. Gentiol (17), isolated from *Gentiana verna*, has a melting point of 215–217° and forms a triacetyl derivative melting at 175–180°.

<sup>3</sup> Referred to by Gintl (12).

It is apparent from the foregoing statements that malol is different from ursin, caryophyllin, and gentiol since malol melts at 285° and yields a diacetyl derivative melting at 199–200°, a monoacetyl derivative melting at 279–281°, and a monomethyl derivative melting at 170.5–171.5°.

It is interesting to note that malol possesses the same general formula,  $C_nH_{2n-12}O_3$ , as two dihydroxy alcohols which were isolated some years ago in the Wellcome Chemical Research Laboratories. Oleanol, melting at 303–304°, was obtained from olive leaves by Power and Tutin (11). Prunol, melting at 275–277°, was isolated from the leaves of *Prunus serotina* by Power and Moore (10). Oleanol and prunol are isomers possessing the formula  $C_{31}H_{50}O_3$ . Malol appears to be the next lower homologue, since it possesses the formula  $C_{30}H_{48}O_3$ .

#### SUMMARY.

For the purpose of examining the constituents of the ether extract of apple skins, mixed material was employed from the closely related varieties of Ben Davis and Black Ben Davis. The following products were obtained:

1. *Triacontane*,  $C_{30}H_{62}$ , melting at 63.5–64°.
2. Fractions of material having the following melting points: 70°, 71–71.5°, 75–76°, 75–77°, 77–78°, 79–79.5°. These fractions were too small in amount for complete identification, but they appeared to consist of hydrocarbons and alcohols or mixtures of these substances.
3. *Heptacosanol*,  $C_{27}H_{56}O$ , melting at 81–81.5° and yielding an acetate melting at 44–46°.
4. *Malol*,  $C_{30}H_{48}O_3$ , a new crystalline alcohol, which crystallizes from absolute alcohol in highly lustrous, prismatic needles, melting at 284–285°. Its *diacetyl derivative*,  $C_{30}H_{46}O_3(\text{CO}\cdot\text{CH}_3)_2$ , and *monoacetyl derivative*,  $C_{30}H_{47}O_3(\text{CO}\cdot\text{CH}_3)$ , form colorless needles which melt, respectively, at 199–200° and about 279–281°. The *monomethyl derivative*,  $C_{30}H_{47}O_3(\text{CH}_3)$ , and *acetyl methyl derivative*,  $C_{30}H_{46}O_3(\text{CH}_3)(\text{CO}\cdot\text{CH}_3)$ , melt at 170.5–171.5° and 243–244°, respectively. Malol forms a crystalline *monosodium salt*,  $C_{30}H_{47}O_3\text{Na}$ , and is dextro-rotatory.

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## A MODIFICATION OF THE FOLIN-WU METHOD FOR MAKING PROTEIN-FREE BLOOD FILTRATES.

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The tungstic acid method introduced by Folin and Wu (1) has proven by far the most desirable one for obtaining blood filtrates free from protein. Folin and Wu direct that 1 volume of blood be first diluted with 7 volumes of distilled water. 1 volume of 10 per cent sodium tungstate is then added, followed by an equal amount of  $\frac{2}{3}$  sulfuric acid.

We have found that the procedure may be simplified by diluting 1 volume of blood with 8 volumes of  $\frac{1}{12}$  sulfuric acid and then adding 1 volume of 10 per cent sodium tungstate. The addition of the reagents in this order requires only two reagents in place of the three needed in the original Folin-Wu procedure. On adding the acid the blood is laked very rapidly and the hemoglobin is changed to acid hematin. No precipitate is obtained until the sodium tungstate is added. The protein is precipitated in a very much more granular form than when the reagents are added in the manner directed by Folin and Wu, filtration is more rapid, a larger amount of filtrate is obtained, and the filtrate is more nearly neutral.

To ascertain whether filtrates so prepared give the same results on analysis as those prepared by the original technique we have determined the non-protein nitrogen constituents, the sugar, and the chlorides on filtrates made from the same sample of blood by the different methods. The amount of filtrate and the reaction have also been determined. The results are shown in Table I. The  $\frac{1}{12}$  acid used was made from the  $\frac{1}{3}$  acid, so exactly the same amount of acid was employed in the two methods.

TABLE I.

No.	Method.	Reaction.										0.1 N NaOH required for 6 cc. filtrate. cc.			
		Non-protein nitrogen.		Uric acid.		Creatinine.		Amino-acid nitrogen.		Chlorides.		Filtrate from 10 cc. blood.	Congo red.	Phenolphtha- lein.	
		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	cc.					
1	Folin-Wu.	34.5	1.3	1.3	4.6	84	415	56					Alkaline.	Acid.	0.25
	Modified.	34.5	1.6	1.3	4.6	74	420	63					"	"	0.20
2	Folin-Wu.	29.4	3.3	1.2	5.4	77	450	55					"	"	0.25
	Modified.	27.3	3.3	1.2	5.5	78	445	65					"	"	0.20
3	Folin-Wu.		2.6			133	470	50					"	"	0.25
	Modified.		2.5			133	470	58					"	"	0.20
4	Folin-Wu.		2.3	1.2	5.3	182	440	49					"	"	0.30
	Modified.		2.3	1.2	5.2	182	450	59					"	"	0.15
5	Folin-Wu.	29.2	2.2	1.2	5.8	89	430	54					"	"	0.30
	Modified.	28.5	2.2	1.2	5.7	89	430	61					"	"	0.20
6	Folin-Wu.	33.8	2.0	1.2	5.6	105	450	59					"	"	0.25
	Modified.	32.6	2.1	1.2	5.5	105	460	63					"	"	0.20
7	Folin-Wu.	57.5	0.9	1.1	4.9	167	560	60					"	"	0.35
	Modified.	60.0	1.0	1.1	5.0	163	560	64					"	"	0.25
8	Folin-Wu.	34.5	2.3			6.2	110	460	54	Slightly acid.			"	"	0.60
	Modified.	34.5	2.3			6.2	108	460	60	Alkaline.			"	"	0.25
9	Folin-Wu.	31.6	0.8	1.2	6.2	94	490	54					"	"	0.30
	Modified.	31.6	1.1	1.2	6.2	98	500	64					"	"	0.20
10	Folin-Wu.	33.0		1.1	8.7	175	460	56					"	"	0.40
	Modified.	33.0		1.1	8.6	182	460	62					"	"	0.25
11	Folin-Wu.	34.0	2.5	1.2	7.0	149	490	51					"	"	0.35
	Modified.	34.0	2.5	1.2	7.0	149	480	62					"	"	0.30
12	Folin-Wu.	37.0	1.8	1.1	8.6	98	430	52					"	"	0.45
	Modified.	37.0	1.7	1.1	8.6	98	440	63					"	"	0.15
Average. {	Folin-Wu.	35.4	2.0	1.2	6.2	122	462	54							0.34
	Modified.	35.3	2.0	1.2	6.2	122	464	62							0.21

The non-protein nitrogen, sugar, and the creatinine have been determined by the method of Folin and Wu (1), the amino-acid nitrogen by the method of Folin (2), the uric acid by the method of Benedict (3), and the chlorides by the technique suggested by Gettler (4).

The filtrate obtained by the modified method averages about 15 per cent more than that obtained by the original technique, where gravity filtration is used, and requires less than two-thirds as much alkali for neutralization. Otherwise the results of the two analyses are practically identical.

It would seem that the only constituent which might be influenced by the addition of acid first would be uric acid. We have found, however, that uric acid can be equally well recovered when added and that blood very low in uric acid will show a slightly higher reading with the modified than with the original technique.

Occasionally a filtrate is obtained which is tinged with the brown of acid hematin. The color does not interfere in any of the determinations. We have used the procedure described in our laboratory for some time and find it of great value where many blood chemical determinations are being made.

#### SUMMARY.

A modification of the Folin-Wu method for the preparation of protein-free blood filtrates is described.

1 volume of blood is diluted with 8 volumes of  $\frac{N}{2}$  sulfuric acid. After laking is complete 1 volume of 10 per cent sodium tungstate is added and the mixture shaken.

The advantages of the method are: (a) only two solutions are required instead of three, (b) filtration is much more rapid, (c) 15 per cent more filtrate is obtained, (d) the filtrate is more nearly neutral.

Parallel determinations of non-protein nitrogen, uric acid, creatinine, amino-acid nitrogen, sugar, and chlorides give practically the same results with filtrates made by the two methods.

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## A STUDY OF THE INORGANIC CONSTITUENTS OF THE BLOOD IN EXPERIMENTAL NEPHRITIS.

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(Received for publication, April 17, 1923.)

During the last decade studies of the histological and functional changes produced in animals poisoned with various nephrotoxic substances have been popular. As regards chemical changes in the blood of such animals it has been shown that accumulations of the various non-protein nitrogenous constituents take place as in the case of acute and chronic nephritis in man (1), and that changes in the alkali reserve (2) are also demonstrable. As yet, however, no account of experimental work dealing with a study of the inorganic blood constituents has appeared. From results obtained in cases of chronic and acute nephritis in man, we know that in these conditions we sometimes (but not invariably), find relatively slight increases in the sodium chloride content of the serum (3) or plasma, marked increases in the amounts of inorganic phosphates (4, 5) and sulfates (6), a decrease in calcium (5), but no alterations in the content of magnesium (5, 6) and potassium (7). So little work has been done on the inorganic constituents of whole blood or corpuscles in these pathological conditions that the field is as yet almost untouched.

In this paper I have collected the results obtained in a series of observations made on rabbits in whom an acute nephritic condition had been induced by the administration of uranium nitrate or sodium tartrate.

This work on experimental nephritis was attempted because it seemed possible that by the selection of nephrotoxic agents which cause a purely tubular or a purely glomerular lesion it might be possible to throw additional light on the selective function of the kidney for inorganic salt. Unfortunately, increasing knowledge

regarding the production of experimental nephritis in animals has led to the belief (8), that most of the injuries produced by the nephrotoxic agents ordinarily employed lead to lesions of mixed type, and I have therefore confined my experiments to observations on animals poisoned with uranium nitrate, which may be taken as an example of a nephritis in which both tubules and glomeruli are involved, and to those treated with sodium tartrate which drug according to the work of Underhill (9) produces purely tubular lesions.

The experimental animals were brought to the laboratory some days before they were to be used and were fed on a diet of oats, carrots, and potatoes. During the period of observation they were kept in metabolism cages and the urine was collected daily, measured, and tested for albumin. The animals were bled from either the heart or the carotid artery after they had first been stunned by a blow on the head.

The analytical methods used were as follows: Sodium and potassium were determined by the methods of Kramer and Tisdall (10), calcium by the method of Clark (11), and magnesium by the procedure described by Denis (12). For the acid radicals the following methods were used: Chlorides were determined by the procedure of Smith (13), phosphates by the Bell-Doisy method (14), and sulfates by the procedure of Denis (15). Non-protein nitrogen was determined by the method of Folin and Wu (16).

As a preliminary to experimental work it has been necessary to obtain figures on the inorganic constituents of rabbit blood and serum as the data to be found in the literature are somewhat scanty. The results obtained by the analysis of the blood of five full grown male rabbits who had fasted for at least 14 hours are collected in Table I. It is, of course, apparent that a more desirable procedure would have been to make a preliminary examination of the blood of the animals in whom nephritis was later to be induced. On account of the relatively large amount of blood needed for the numerous determinations made this procedure proved impossible of application in this work, as it was found that the rabbits almost invariably died after the preliminary bleeding.

The averages of the results of the analyses of normal rabbit's blood differ somewhat from the figures published by Abderhalden

(17); this divergence is most striking in the case of the sodium content of serum which is somewhat lower than the figure given by him (355 as against 444 mg.) and the magnesium values in both whole blood and serum which are not more than half the value of those given by this investigator. The average value for serum potassium, 19.9 mg., is also somewhat lower than the figure, 25.9 mg., given by Abderhalden. It is possible that this investigator did not separate the corpuscles and serum as promptly as was done

TABLE I.

*Inorganic Constituents of the Blood and Serum of Fasting Rabbits.*

Rabbit No	Per 100 cc. serum.							Per 100 cc. whole blood.																
	Na		K		Ca		Mg		Cl		Inorganic PO <sub>4</sub> as P		SO <sub>4</sub> as S.		Na		K		Ca		Mg		SO <sub>4</sub> as S.	
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	352	20.0	10.0	3.0	378	4.1	3.2	250	171	5.4	2.2	2.3												
2	347	18.6	10.1	2.6	348	4.1	3.9	251	196	5.0	2.4	2.1												
3	356	19.6	9.8	2.8				285	195	5.2	2.5													
4	350	19.5	10.1	2.7	360	4.0	3.9	286	151	6.3	2.4	2.0												
5	374	22.2	10.0	3.3	368	4.4	3.0	280	176	4.3		2.0												
Average....	355	19.9	10.0	2.8	363	4.2	3.5	266	178	5.2	2.4	2.1												

in this work, a fact which would account for the higher potassium values found by him.

In Experiments 1 to 4, the animals were given a large dose of uranium and were killed from 48 to 72 hours after the injection. By this procedure it was hoped that we would be able to obtain indications of any changes which might take place in the inorganic constituents of the blood in the early and most acute stage of poisoning. In Experiments 5 to 7 a smaller dose of uranium was used in order that the animals might be kept alive for a greater length of time than in the earlier experiments, and in Experiments 8 and 9 sodium tartrate was administered in place of uranium nitrate.

*Experiment 1.*

Rabbit 3. Male. Weight 2,300 gm.

June 5, 1922, 8.30 a.m. Injected subcutaneously 6 mg. of  $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ .

June 8, 1922, 9.00 a.m. Bled from heart.

	Per 100 cc.	
	Blood.	Serum.
	mg.	mg.
Non-protein N.....	205	
$\text{PO}_4$ (as P).....	10	3.1
Cl.....		366
$\text{SO}_4$ (as S).....	14.0	16.1
Na.....	29.0	34.8
K.....	167.0	23
Ca.....	4.0	10.2

*Experiment 2.*

Rabbit 4. Male. Weight 2,240 gm.

June 5, 8.40 a.m. Injected subcutaneously 6 mg. of  $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ .  
" 8.9.20 a.m. Bled from heart.

	Per 100 cc.	
	Blood.	Serum.
	mg.	mg.
Non-protein N.....	200	
$\text{PO}_4$ (as P).....	9.0	4.4
Cl.....		316
$\text{SO}_4$ (as S).....	22	25
Na.....	290	388
K.....	200	25
Ca.....	4.2	10.0
Mg.....		2.6

*Experiment 3.*

Rabbit 7. Male. Weight 2,600 gm.

June 8, 1922. Injected 6 mg. of  $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ .

" 10. Bled from heart.

	Per 100 cc.	
	Blood.	Serum.
	mg.	mg.
Non-protein N.....	192	
$\text{PO}_4$ (as P).....	12.0	3.0
Cl.....		322
$\text{SO}_4$ (as S).....	16.2	18
Na.....	280	
K.....	175	24
Ca.....	4.1	11
Mg.....		2.5

*Experiment 4.*

Rabbit 6. Male. Weight 2,900 gm.

June 8, 9.00 a.m. Injected subcutaneously 6 mg. of  $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ .

" 10. Bled from heart.

	Per 100 cc.	
	Blood.	Serum.
	mg.	mg.
Non-protein N.....	200	
$\text{PO}_4$ (as P).....		3.1
Cl.....		33.9
$\text{SO}_4$ (as S).....	12.3	14
Na.....	250	380
K.....		23
Ca.....	4.2	11
Mg.....		2.5

*Experiment 5.*

Rabbit 10. Female. Weight 1,660 gm.

Dec. 4, 1922, 4.00 p.m. Injected subcutaneously 3 mg. of  $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ .

Dec. 11, 9.30 a.m. Bleed from carotid artery.

On Dec. 5 urine containing a large trace of albumin was excreted. The animal continued to eat as usual during the remainder of the experiment.

	Per 100 cc.	
	Blood.	Serum.
	mg.	mg.
Non-protein N.....	96	
$\text{PO}_4$ (as P).....		3.3
Cl.....	2.1	
$\text{SO}_4$ (as S).....		2.6
Na.....	263	
K.....	208	24.0
Ca.....	5.3	10.0
Mg.....	3.3	2.9

*Experiment 6.*

Rabbit 13. Female. Weight 2,770 gm.

Dec. 13, 1922, 1.30 p.m. Animal injected subcutaneously with 3 mg. of  $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ .

On Dec. 14 the urine contained albumin which continued to be excreted in increasing amounts until Dec. 19 at 9.00 a.m. when the animal was bled from the carotid artery. This animal continued to eat in a normal manner throughout the experiment.

	Per 100 cc.	
	Blood.	Serum.
	mg.	mg.
Non-protein N.....	30	
$\text{PO}_4$ (as P).....		4.0
Cl.....		339
$\text{SO}_4$ (as S).....	2.75	3.1
Na.....	276	400
K.....	178	19.7
Ca.....	7	9.7
Mg.....		

*Experiment 7.*

Rabbit 14. Female. Weight 2,680 gm.

Dec. 13, 1922, 1.40 p.m. Animal injected subcutaneously with 3 mg. of  $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ .

On Dec. 14 it was noted that the urines contained a large trace of albumin. The animal continued to excrete albumin and to eat all food offered until she was bled at 9.30 a.m. on Dec. 19.

	Per 100 cc.	
	Whole blood.	Serum.
	mg.	mg.
Non-protein N.....	30	
P (inorganic).....		4.35
Cl.....		381
SO <sub>4</sub> (as S).....	2.8	
Na.....	285	392
K.....	186	18.1
Ca.....		10.9
Mg.....	6.1	

*Experiment 8.*

Rabbit 11. Male. Weight 1,903 gm.

Dec. 11, 1922. The animal, which had been without food for 24 hours, was injected subcutaneously with 2 gm. of Kahlbaum's tartaric acid which had been neutralized with Na<sub>2</sub>CO<sub>3</sub>. The total volume of liquid injected amounted to 12 cc. On Dec. 12 the animal refused food and passed urine containing a large trace of albumin. On Dec. 13 he was bled from the carotid artery.

	Per 100 cc.	
	Blood.	Serum.
	mg.	mg.
Non-protein N.....	88	
PO <sub>4</sub> (as P).....		5.0
Cl.....		
SO <sub>4</sub> (as S).....	3.5	
Na.....	213	287
K.....	203	18.6
Ca.....	5.4	9.6
Mg.....	2.2	1.7

*Experiment 9.*

Rabbit 12. Female. Weight 1,849 gm.

Dec. 12, 9.45 a.m. The animal, which had been kept without food for 24 hours, was injected subcutaneously with 1.2 gm. of Kahlbaum's tartaric acid neutralized with  $\text{Na}_2\text{CO}_3$ , and made up to a volume of 8 cc. During the night of Dec. 12 the animal passed urine containing a large trace of albumin and on Dec. 13 at 9.45 a.m. she was bled from the carotid artery.

	Per 100 cc.	
	Blood.	Serum.
	mg.	mg.
Non-protein N.....	50	
P.....		4.5
Cl.....		
SO <sub>4</sub> (as S).....	2.7	
Na .....	222	362
K.....	241	20.2
Ca.....	5.1	11.2
Mg.....	2.0	1.7

An inspection of the results presented in Experiments 1 to 9 indicates that although in every case but two (Experiments 6 and 7), there was found a considerable increase in the non-protein nitrogen of the blood no constant retention of any inorganic component was noted except in the case of the sulfate fraction in which very large increases were found in the first four experiments in which the dosage of uranium had been high. In the case of the other animals in whom a less acute condition prevailed (as judged by the relative degree of nitrogen retention) practically no retention of even the sulfate ion could be demonstrated.

The fact has been definitely established that in certain cases of human nephritis there is a marked increase in the concentration of inorganic phosphates in the serum and a corresponding decrease in calcium, but in these experiments no suggestion of such a condition was noted.

The results on whole blood are essentially similar to those obtained on serum, *viz.* a retention of sulfate is noted in the most severe cases, but the concentrations of all the other constituents, including potassium, show only slight variations with no indications of even a slight degree of retention.

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# CHEMICAL STUDY OF SEVERAL MARINE MOLLUSKS OF THE PACIFIC COAST.

## THE REPRODUCTIVE SYSTEM.\*

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### INTRODUCTION.

The mature male reproductive system of the abalone, limpet, *Cryptochiton*, and *Ischnochiton* can readily be distinguished from that of the female by its cream color, that of the female being green. No such sexual distinction can be made in the Pismo clam. Very little attention from a chemical standpoint has been paid to the reproductive system of marine invertebrates. The only case reported, as far as the chemical study is concerned, is the analysis of the spermatozoa of the sea urchin by Mathews under the direction of Kossel.

The same species of mollusks, namely the abalone, Pismo clam, *Cryptochiton*, and *Ischnochiton*, and methods of analysis as in previous investigations were employed for this study by the author.<sup>1</sup>

Sufficient quantities of material could not be gathered from the owl limpet to make a complete study of its reproductive system.

The results of these analyses are given in Tables I and II.

As the animals were collected about the time of the breeding season, the reproductive system was considerably enlarged and compactly filled with sperm and eggs. No special study of male and female was carried on except in determining the protein

\* This paper is a part of a thesis to the Department of Chemistry of Stanford University in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

<sup>1</sup> Albrecht, P. G., *J. Biol. Chem.*, 1920-21, xlv, 395.

TABLE I.  
*Reproductive System.*

Constituents.	Abalone.	Pismo clam.	Crypto- chiton.	Iachno- chiton.
100 gm. of fresh reproductive system tissue.				
Water.....	74.20	65.40	74.40	79.20
Total solids.....	25.80	34.60	25.60	20.80
Ash.....	5.46	4.22	6.46	7.52
Alcohol extractives, F1 + F2.....	10.06	12.20	11.61	10.10
Lipin fraction, F1.....	3.79	3.50	8.77	5.20
Water-soluble fraction, F2.....	6.27	8.70	2.84	4.80
Alcohol- and water-insoluble fraction, F3.....	15.71	22.40	14.00	10.70
Ash of F1 + F2.....	0.76	1.28	0.86	1.30
" " F3.....	4.70	2.94	5.40	6.22
Total N.....	4.56	3.04	1.99	2.00
Protein.....	29.05	19.90	12.65	12.70
Purine N.....	None.	None.	None.	None
Creatine and creatinine.....	"	"	"	"
Uric acid.....	"	"	"	"
100 gm. of F1 + F2 + F3.				
Alcoholic extracts, F1 + F2.....	39.9	35.3	45.4	48.1
Alcohol-insoluble residue, F3.....	60.1	64.7	54.6	51.9
Total solids, F1.....	37.7	28.7	75.5	52.0
" " F2.....	62.3	71.3	24.5	48.0
Ash of F1 + F2.....	12.1	14.7	30.3	27.1
" " F3.....	30.0	18.1	38.0	58.1
100 gm. of F1.				
Total N.....	0.74	0.91	0.65	0.60
Amino-acid N.....	0.072	0.061	0.160	0.027
Total sulfur.....	0.36	0.51	0.80	0.15
" phosphorus.....	0.76	1.11	0.67	0.52
Lipin sugar.....	None.	None.	None.	None.
100 gm. of F2.				
Total N.....	7.63	4.08	10.09	4.11
Amino-acid N.....	4.50	1.44	4.30	1.20
Proteose N.....	0.41	0.25	1.06	0.62
Urea N.....	None.	None.	0.11	0.12
" .....	"	"	0.30	0.30
Ammonia.....	0.41	0.23	1.26	0.46
Total sulfur.....	6.25	2.30	7.05	5.00
Inorganic sulfur.....	0.35	0.11	0.64	0.25

TABLE I.—*Concluded.*

Constituents.	Abalone.	Pismo clam.	Crypto-chiton.	Ischnochiton.
100 gm. of F2—Concluded.				
Total phosphorus.....	0.35	0.29	1.59	0.32
Inorganic phosphorus.....	0.031	0.026	0.180	0.191
Creatine and creatinine.....	None.	None.	None.	None.
Uric acid.....	"	"	"	"
Reducing sugars after hydrolysis.....	29.09	6.55	3.91	4.75
100 gm. of F3.				
Total N.....	15.65	4.49	15.1	9.13
Protein ( $\times 6.37$ ).....	97.65	28.11	94.6	57.15
Total phosphorus.....	1.77	0.67	2.06	1.92
Phospho-protein phosphorus.....	0.16	0.13	0.22	0.21
Total sulfur.....	1.11	0.77	1.03	1.29
Creatine and creatinine.....	None.	None.	None.	None.
Uric acid.....	"	"	"	"
Reducing sugars after hydrolysis.....	0.46	18.5	4.07	0.86

Inorganic constituents per 100 gm. of dry tissue.

SiO <sub>2</sub> .....	0.47	0.66	2.56	1.20
Fe <sub>2</sub> O <sub>3</sub> .....	1.71	2.16	1.92	1.55
CaO.....	0.48	0.63	1.08	1.00
MgO.....	0.32	0.47	0.32	0.30

TABLE II.  
*Enzymes of the Reproductive System.*

	Abalone.	Pismo clam.	Crypto-chiton.	Ischnochiton.	Owl limpet.
Amylase.....	+	+	+	+	+
Cytase.....	-	-	-	-	-
Emulsin.....	-	+	+	Trace.	-
Glycogenase.....	+	+	+	+	+
Lactase.....	-	Trace.	-	-	-
Lipase.....	+	+	-	-	+
Maltase.....	+	+	+	+	+
Protease.....	-	-	-	-	-
Sucrase.....	-	-	-	-	-
Urease.....	+	+	-	-	+
Uricase.....	-	-	-	-	-

content of the reproductive system of the two sexes. The results (Table III) show that the male reproductive system, except in the cryptochiton, has a higher amount of total nitrogen.

TABLE III.  
*Relative Total Nitrogen Content in the Male and Female Abalone  
and Cryptochiton.*

	per cent	per cent
Abalone, red, male.....	4.84	4.72
"    "    female.....	4.28	4.49
"    black, male.....	4.76	4.90
"    "    female.....	2.66	2.80
Pismo clam.....	2.94	3.15
Cryptochiton, male.....	1.54	1.82
"    "    female.....	2.45	2.14
Ischnochiton, male.....	1.82	2.17
"    female was not obtainable at that time.		

#### DISCUSSION.

The large amount of lipin substance (Table I) present, is worthy of note. The alcohol-ether extract compares fairly well with the findings of Mathews, on the sea urchin. He found the reproductive system of this animal to contain 49.91 per cent of lipins.

The absence of urea in abalone and Pismo clam and the presence of urease in this organ, are worthy of note. Ammonia is present in all of the reproductive organs, but leading in amount in cryptochiton. Glycogen is apparently absent in the organ, but large amounts of reducing sugars are found in the alcoholic extract as well as in the residue. These sugars do not necessarily come from glycogen. Glycoprotein evidently present in considerable amounts is readily hydrolyzed to reducing sugars. Uric acid, creatine, and creatinine are absent.

Of inorganic substances, iron, which is so important in molluscan liver is the chief constituent. That enzymes are present in the reproductive gland has been reported by Abelous and Heim.<sup>2</sup> In their experiments on enzymes in the reproductive glands the authors noticed a comparative weakness as compared with the strength of the enzymes in liver and digestive fluid. The sugar-

<sup>2</sup> Abelous, J. E., and Heim, F., *Compt. rend. Soc. biol.*, 1891, xlvi, 273.

splitting enzymes, glycogenase, invertase, and maltase were detected. No extensive investigation on glycogen in this gland has been carried on, but I believe that glycogen is present in noticeable amounts during certain seasons of the year. My efforts to find it in these specimens have, however, been fruitless. Emulsin, the enzyme which decomposes amygdalin, probably serves here the purpose of splitting a phosphatide-glucoside.

The absence of lipase in this organ is not surprising. The common observation that tissues rich in fats are poor in lipases applies here. We notice the absence of lipase especially in the organs of *Cryptochiton* and *Ischnochiton*, where the amount of fat is high.

#### SUMMARY.

The reproductive system in all forms is high in lipoids.

Glycogen is absent. Large amounts of reducing sugars were found not only upon hydrolysis of the alcohol-insoluble, but also of the alcohol-soluble residue.

Urea was found only in the *Cryptochiton* and *Ischnochiton*, its absence from the abalone and Pismo clam in all probability being due to the urease which was found in these forms.

Enzymes are present in considerable number, amylase, glycogenase, and maltase being found in all five forms, emulsin in the Pismo clam and cryptochiton, and lipase and urease in abalone, clam, and limpet.



## THE SYNTHESIS OF "BIOS" BY YEAST GROWN IN A SOLUTION OF PURIFIED NUTRIENTS.

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It has been shown in feeding tests with young rats that yeast grown in a distilled water solution of sucrose and the necessary mineral salts may furnish the vitamin B essential for growth (1, 2). It has also been found in tests made in this laboratory that pigeons which had developed polyneuritis on a diet of polished rice, were "cured" by the administration of this yeast. Heller has recently reported similar results with "synthetic" yeast (3). These findings establish beyond a doubt the ability of yeast to synthesize vitamin B when growing in vitamin-free media. The results obtained in the feeding tests and the conclusions drawn from them are at variance with those of Eijkman and his coworkers (4), who, after much experience in yeast experimentation, expressed the conviction that the yeast cell gains its antineuritic factor as such from the culture medium and cannot, strictly speaking, synthesize it.

The subject of yeast growth and stimulation was given a new interest in 1919 by the publication of methods for the detection and estimation of water-soluble B, using yeast as a test organism (5, 6). These methods were based on the supposition that Wildiers' "bios" and vitamin B are identical. Attempts to apply these methods failed of their original intent, but brought to light a number of interesting facts.

In their use of Williams' method Souza and McCollum (7) found that alkalinized material, inactive for rats, gave excellent results in stimulating yeast. Later Fleming (8) reported that an extract of unmilled rice produced as great an increase in the growth of yeast after treatment with alkali to destroy vitamin B as before

treatment. Emmett and Stockholm (9) used 95 per cent alcohol extracts of heated and autoclaved unmilled rice in tests with yeast, pigeons, and rats. Their conclusion was that the yeast growth-promoting factor is not the antineuritic vitamin nor does it stimulate the growth of young rats.

Through the practical application of the yeast test for the antiberi-beri vitamin Funk and Dubin (10) were led to question whether there was only a single vitamin present in their autolyzed yeast extract. Later they were able to report an almost quantitative separation of the yeast growth stimulant from the vitamin curative for beri-beri by means of absorption with fuller's earth and norit (11, 12).

As is well known small amounts of a water solution of commercial peptone or of Liebig's extract of beef very markedly affect yeast growth. Wildiers mentions them along with wort as sources of "bios" (13). Both peptone and beef extract are devoid of vitamin B.

Heaton (14) has called attention to the contrasting distribution of "bios" and the antineuritic vitamin among the organs of animals. He also found "bios" in the organs of polyneuritic pigeons and of rats fed a diet free from water-soluble B. He is of the opinion that the activating substance for yeast is not the antineuritic vitamin B.

Because of the peculiar nature of the problem it has been difficult either to prove or to disprove the original assumption of Williams. The evidence cited would, however, indicate that vitamin B and "bios" are not identical. It seemed, therefore, of interest to test the ability of yeast to synthesize its own growth-promoting substance by ascertaining the action of extracts of yeast grown in Nutrient Solution 3 on the proliferation of yeast cells in a medium of purified nutrients. Inasmuch as a review of the literature from 1901 disclosed no uniform method for the production of yeast or for the measurement of the effect of added substances on its proliferation or fermentative activity, it was decided for the major part of the present investigation to use a procedure described in detail in a former paper (1), and to add to Nutrient Solution 3 measured quantities of extracts of yeast grown in different media as well as extracts of malt, wheat germ, and other materials which cause an increased production of yeast cells when added to the sucrose-mineral salts solution.

In the preparation of the extracts 4 gm. of air-dry material were extracted with 100 cc. of the solvent. The solvents used were ether, chloroform, absolute alcohol, 80 per cent alcohol (recommended by Wildiers), and 40 per cent alcohol. Extracts were also prepared by the Osborne and Wakeman (15) method for yeast extract, using 100 cc. of distilled water to which 1 cc. of 1 per cent acetic acid had been added. Ether-extracted wheat germ was used for making the extracts with other solvents, but none of the other materials was first extracted with ether. Just before using 25 cc. of the filtered alcohol, ether or chloroform extracts were carefully evaporated to dryness in a silica milk dish on the water bath and the residue was taken up in 10 cc. of distilled water. This solution was filtered and heated to boiling. The acetic acid of the water extracts was neutralized with ammonia, and the volume regulated so that 10 cc. were equivalent to 1 gm. of material. Measured quantities of the sterile extract were added to 150 cc. of Nutrient Solution 3 in the production flasks.

*Nutrient Solution 3.*

1	liter distilled water.
50	gm. cane-sugar (Domino).
2	" potassium dihydrogen phosphate, c.p.
2.35 "	ammonium sulfate, c.p.
0.25 "	calcium chloride, c.p.
0.25 "	magnesium sulfate, c.p.

The suspensions of cells used for seeding the production flasks were from yeasts which had been growing with fresh inoculations every week for 2 years in Nutrient Solution 3. 1 cc. of a suspension of yeast cells, containing from 0.0014 to 0.0018 gm. of yeast if weighed air-dry, was added to 150 cc. of sterile medium in a liter Erlenmeyer flask. In some cases the same suspension was used to inoculate two or three sets of ten flasks each. In all cases the seedings were uniform for any one set, which consisted of a control flask without extract and four flasks with increasing amounts of extract. The sets were made up in duplicate. The yeast was grown at room temperature which varied considerably, and to this is due in part the variation to be noted in the yield of yeast in the control flasks of the same variety of yeast in different

experiments. But all the flasks of any one set were exposed to exactly the same conditions. On the 8th day after seeding, the yeast of each flask was filtered, washed, dried at room temperature, and weighed.

Tables I to V present the experimental data.

From an inspection of Table I it is apparent that with the addition of extracts of "synthetic" yeast in graduated amounts there is a corresponding increase in the yield of yeast. The relation is not quantitative, however.

The results in Table I compare very favorably with those for commercial yeasts in Table II, and with those for wheat germ and malt in Table III. The extracts of polished rice and white flour are without effect on yeast growth.

The absolute alcohol extracts, Table IV, show little or no effect, while the 80 per cent alcohol extracts of the residues from the absolute alcohol extraction make a better showing. In general the 80 per cent alcohol extracts gave the best results.

The greatest increase to be noted is that of the 1 per cent water solution of Liebig's extract of beef in Table V, the weight of yeast in the control flask being approximately half of that in the flask to which 4 cc. of the extract were added.

It is evident from the experimental data presented that the variation in the yield of yeast with and without extract may be taken as indicative of the efficiency of any one extract, and the increase in the yield of yeast in the different sets furnishes a qualitative basis of comparison of the different extracts. One is not justified in believing this method can be made the basis of a quantitative comparison of the relative stimulative value of different extracts on yeast.

Conditions are, in all probability, not the same in synthetic media in the case of seedings of yeast too small to be followed by a demonstrable multiplication of cells, and in the case of seedings large enough to insure growth. Increasing the yield of yeast by the addition of extracts to the medium may conceivably be quite different from effecting a multiplication of cells by the same agents. The data so far presented are from comparatively large seedings, containing approximately 1.5 mg. of dry yeast cells per 1 cc. of suspension. The controls show a yield of yeast varying from 0.115 to 0.229 gm. It seemed advisable, therefore, to make

TABLE I.  
Extracts of Yeast Grown in Nutrient Solution 3.

	Air-dry yeast.			Air-dry yeast.			
	<i>Saccharomyces ellipsoideus.</i>			<i>Saccharomyces ellipsoideus.</i>			
	1	2	cc.	gm.	gm.	cc.	
80 per cent alcohol extract of <i>Saccharomyces ellipsoideus.</i>	0 $\frac{1}{2}$ 1 2 4	0.147 0.165 0.173 0.224 0.250	0.151 0.172 0.184 0.191 0.246	40 per cent alcohol extract of <i>Saccharomyces ellipsoideus.</i>	0 $\frac{1}{2}$ 1 2 4	0.147 0.149 0.165 0.195 0.205	0.150 0.156 0.170 0.200 0.212
	<i>Saccharomyces cerevisiae.</i>			<i>Saccharomyces cerevisiae.</i>			
	1	2	cc.	gm.	gm.	cc.	
	0 $\frac{1}{2}$ 1 2 4	0.202 0.240 0.2558 0.290 0.310	0.175 0.205 0.229 0.221 0.300	40 per cent alcohol extract of <i>Saccharomyces cerevisiae.</i>	0 $\frac{1}{2}$ 1 2 4	0.199 0.211 0.213 0.219 0.228	0.198 0.200 0.218 0.227 0.238
	Yeast XII.			Yeast XII.			
	1	2	cc.	gm.	gm.	cc.	
	0 $\frac{1}{2}$ 1 2 4	0.183 0.190 0.2066 0.280 0.299	0.167 0.185 0.191 0.194 0.284	40 per cent alcohol extract of <i>Saccharomyces cerevisiae.</i>	0 $\frac{1}{2}$ 1 2 4	0.150 0.199 0.190 0.288 0.297	0.170 0.172 0.2196 0.288 0.291
	Yeast XII.			Yeast XII.			
	1	2	cc.	gm.	gm.	cc.	
	0 $\frac{1}{2}$ 1 2 4	0.116 0.122 0.153 0.208 0.270	0.123 0.126 0.144 0.150 0.2334	40 per cent alcohol extract of Yeast XII.	0 $\frac{1}{2}$ 1 2 4	0.156 0.176 0.206 0.251 0.292	0.179 0.200 0.209 0.227 0.289
	<i>Saccharomyces ellipsoideus.</i>			<i>Saccharomyces cerevisiae.</i>			
	1	2	cc.	gm.	gm.	cc.	
	0 $\frac{1}{2}$ 1 2 4	0.2297 0.2856 0.3208 0.3542 0	0.2200 0.2829 0.3324 0.3407 0.2297	Osborne and Wakeman's extract of <i>Saccharomyces ellipsoideus.</i>	0 $\frac{1}{2}$ 1 2 4	0.2129 0.2447 0.2607 0.2601 0.3063	0.2093 0.2364 0.2558 0.2783 0.3148

a few tests with seedings too small to give a measurable increase in Nutrient Solution 3 without extract and to add to corresponding flasks 80 per cent alcohol extracts of *Saccharomyces ellipsoideus*, *Saccharomyces cerevisiae*, and yeast XII grown in the same synthetic

TABLE II.  
Extracts of Commercial Yeasts.

	Air-dry yeast.				Air-dry yeast.			
	<i>Saccharomyces ellipsoideus.</i>		<i>Saccharomyces cerevisiae.</i>		<i>Saccharomyces ellipsoideus.</i>		<i>Saccharomyces cerevisiae.</i>	
	1	2	1	2	1	2	1	2
cc.	gm.	gm.	cc.	gm.	gm.	cc.	gm.	gm.
80 per cent alcohol extract of Fleischmann's yeast cake.	0 ½ 1 2 4	0.174 0.180 0.200 0.288 0.330	0.187 0.205 0.223 0.303 0.355	Osborne and Wakeman's extract of North-western Yeast Company's yeast.	0 ½ 1 2 4	0.209 0.219 0.232 0.244 0.256	0.212 0.226 0.242 0.248 0.266	
80 per cent alcohol extract of North-western Yeast Company's yeast.	0 ½ 1 2 4	0.1792 0.2356 0.2620 0.2733 0.2595	0.1887 0.2355 0.279 0.3073 0.272	40 per cent alcohol extract of Fleischmann's yeast cake.	0 ½ 1 2 4	0.188 0.244 0.245 0.292 0.302	0.204 0.218 0.224 0.275 0.306	
Osborne and Wakeman's extract of Fleischmann's yeast cake.	0 ½ 1 2 4	0.208 0.245 0.267 0.290 0.315	0.200 0.232 0.248 0.290 0.310	40 per cent alcohol extract of North-western Yeast Company's yeast.	0 ½ 1 2 4	0.2047 0.265 0.271 0.287 0.298	0.213 0.258 0.264 0.274 0.292	

solution. The method employed was essentially that of Funk and Dubin (10), modified somewhat by Eddy and his coworkers (16).

A measurable increase of yeast cells occurred invariably only in the flasks to which extracts had been added, the results coin-

TABLE III.  
Extracts of Grain Products.

	Air-dry yeast			Air-dry yeast.			
	Yeast XII.			Yeast XII			
	1	2		1	2		
80 per cent alcohol extract of wheat germ.	cc.	gm.	gm.	cc.	gm	gm.	
	0	0.206	0.202	40 per cent alcohol extract of wheat germ.	0	0.174	0.170
	$\frac{1}{2}$	0.248	0.2345		$\frac{1}{2}$	0.173	0.191
	1	0.270	0.250		1	0.2356	0.2013
	2	0.2960	0.274		2	0.245	0.304
	4	0.354	0.3592		4	0.3268	0.323
	<i>Saccharomyces cerevisiae.</i>			<i>Saccharomyces cerevisiae.</i>			
	1	2		1	2		
80 per cent alcohol extract of malt.	0	0.195	0.2085	40 per cent alcohol extract of malt.	0	0.191	0.168
	$\frac{1}{2}$	0.255	0.260		$\frac{1}{2}$	0.195	0.208
	1	0.260	0.243		1	0.232	0.246
	2	0.295	0.271		2	0.263	0.265
	4	0.328	0.3244		4	0.299	0.303
	Yeast XII.			Yeast XII.			
	1	2		1	2		
80 per cent alcohol extract of polished rice.	0	0.282	0.280	40 per cent alcohol extract of polished rice.	0	0.252	0.267
	$\frac{1}{2}$	0.291	0.2974		$\frac{1}{2}$	0.260	0.260
	1	0.2645	0.285		1	0.265	0.272
	2	0.277	0.291		2	0.270	0.260
	4	0.2636	0.2924		4	0.268	0.255
	<i>Saccharomyces cerevisiae.</i>			<i>Saccharomyces cerevisiae.</i>			
	1	2		1	2		
80 per cent alcohol extract of white flour.	0	0.246	0.242	40 per cent alcohol extract of white flour.	0	0.2139	0.2085
	$\frac{1}{2}$	0.247	0.247		$\frac{1}{2}$	0.2187	0.219
	1	0.2464	0.241		1	0.212	0.215
	2	0.240	0.244		2	0.224	0.221
	4	0.251	0.250		4	0.206	0.225
	Yeast XII.						
	1	2					
Osborne and Wake- man's extract of wheat germ.	0	0.184	0.209				
	$\frac{1}{2}$	0.228	0.300				
	1	0.319	0.324				
	2	0.347	0.348				
	4	0.371	0.354				

## Synthesis of "Bios" by Yeast

ciding with those from the larger seedings. The addition of extracts of yeast grown in Nutrient Solution 3 was followed by a proliferation of cells that did not occur in the controls.

TABLE IV.  
*Absolute Alcohol Extracts.*

	Air-dry yeast.			Air-dry yeast.			
	<i>Saccharomyces cerevisiae.</i>			<i>Saccharomyces cerevisiae.</i>			
	1	2		1	2		
Absolute alcohol extract of <i>Saccharomyces cerevisiae.</i>	cc.	gm.	gm.	cc.	gm.	gm.	
	0	0.1865	0.184	80 per cent alcohol extract of residue from absolute alcohol extract.	0	0.176	0.1755
	$\frac{1}{2}$	0.198	0.193		$\frac{1}{2}$	0.1979	0.2085
	1	0.2035	0.209		1	0.2156	0.2236
	2	0.218	0.2175		2	0.2313	0.235
Absolute alcohol extract of <i>Saccharomyces ellipsoideus.</i>	4	0.220	0.2256		4	0.2394	0.236
	Yeast XII.			Yeast XII			
	1	2		1	2		
	0	0.134	0.139	80 per cent alcohol extract of residue from absolute alcohol extract of <i>Saccharomyces ellipsoideus.</i>	0	0.114	0.1005
	$\frac{1}{2}$		0.151		$\frac{1}{2}$	0.136	0.117
Absolute alcohol extract of Fleischmann's yeast cake.	1	0.140	0.151		1	0.139	0.127
	2	0.144	0.135		2	0.1385	0.145
	4	0.145	0.160		4	0.2345	0.2390
	<i>Saccharomyces cerevisiae.</i>			<i>Saccharomyces cerevisiae.</i>			
	1	2		1	2		
Absolute alcohol extract of Fleischmann's yeast cake.	0	0.1775	0.1826	80 per cent alcohol extract of residue from absolute alcohol extract of Fleischmann's yeast cake.	0	0.148	0.146
	$\frac{1}{2}$	0.1856	0.176		$\frac{1}{2}$	0.182	0.1744
	1	0.174	0.186		1	0.174	0.1908
	2	0.174	0.1684		2	0.215	0.206
	4	0.196	0.193		4	0.2535	0.2396

TABLE V.  
Other Extracts.

	Air-dry yeast.			Air-dry yeast.			
	<i>Saccharomyces cerevisiae.</i>			<i>Saccharomyces cerevisiae.</i>			
	cc.	gm.	gm.	cc.	gm.	gm.	
80 per cent alcohol extract of peptone (Difco).	0	0.2289	0.194	1 per cent water solution of peptone (Difco).	0	0.206	0.213
	$\frac{1}{2}$	0.2744	0.265		$\frac{1}{2}$	0.242	0.250
	1	0.281	0.282		1	0.258	0.262
	2	0.297	0.304		2	0.287	0.290
	4	0.351	0.365		4	0.340	0.353
40 per cent alcohol extract of autoclaved steak.	<i>Saccharomyces cerevisiae.</i>			<i>Saccharomyces cerevisiae.</i>			
	1	2		1	2		
	0	0.196	0.183	1 per cent water solution of Liebig's extract of beef.	0	0.179	0.190
	$\frac{1}{2}$	0.176	0.185		$\frac{1}{2}$	0.179	0.220
	1	0.1865	0.201		1	0.230	0.256
	2	0.187	0.199		2	0.297	0.291
	4	0.239	0.2604		4	0.353	0.359

Neither chloroform nor ether extracts gave any increase in yield.

#### DISCUSSION.

Experiments have been conducted with three varieties of yeast, *Saccharomyces cerevisiae*, *Saccharomyces ellipoideus*, and yeast XII.

Data are presented which show that there is an increased production of yeast in a sucrose-mineral salts medium to which alcohol or water extracts of the above named yeasts, commercial yeasts, wheat germ, malt, peptone, Liebig's extract of beef, and autoclaved steak have been added.

It is shown conclusively that extracts of yeasts grown in a distilled water solution of pure sugar and nutrient salts, serve to stimulate the growth of seedlings in such medium. The stimulation is comparable in effectiveness with that of extracts of commercial yeasts such as Fleischmann's yeast cake and the dry yeast of the Northwestern Yeast Company, as indicated by the data of Tables I and II.

It seems necessary to interpret these results as signifying that yeast synthesizes a substance, under the experimental conditions

described, which stimulates the growth of the culture to which it is added. This substance is without doubt the "bios" of Wildiers and, until its chemical identity is established, should be so designated. Small seedings of yeast cells in the new environment of a fresh inoculation in a synthetic medium grow very slowly, but proliferate much more rapidly when the nutrient principle "bios" is supplied. It appears, therefore, that "bios" does not function in the manner of a vitamin since development in an animal is impossible in the absence of an indispensable nutrient principle of this class which the organism does not elaborate. Rather "bios" appears to be a substance which, while capable of synthesis by the yeast cell, is formed with some difficulty. Yeast cells developing slowly in a "bios"-free nutrient solution accumulate the substance, so that extracts made from them accelerate the growth of seedlings by providing it in abundance.

The experimental data set forth in this paper make it clear that the growth-stimulating substance for yeast which Funk and Dubin have recently discussed cannot logically be placed in the classification of the vitamins. It is not an indispensable nutrient principle for yeast, since it is synthesized during the slow proliferation of yeast cells in a medium of purified nutrients.

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## STUDIES ON THE DIGESTIBILITY OF PROTEINS IN VITRO.

### IV. ON THE DIGESTIBILITY OF THE COTTONSEED GLOBULIN AND THE EFFECT OF GOSSYPOL UPON THE PEPTIC- TRYPTIC DIGESTION OF PROTEINS.

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Various studies on the digestibility of the protein in cottonseed meals and flours have been reported (1 to 4). All the values given are decidedly below those of the meat proteins and casein, the average being about 83 per cent. The isolated cottonseed globulin, on the other hand, has been shown by Osborne and Mendel (5) to be capable of supporting growth at quite the normal rate. This suggests that the cottonseed globulin may be more readily digested when isolated and fed as the protein constituent of an artificial diet than when fed in combination with the other substances with which it is associated in the cottonseed press-cake preparations. This theory has, in fact, already been put forward by Alsberg and Schwartze (6), on the basis of experiments in which they were able to demonstrate that the addition of gossypol to meat fed to cats resulted in an increased fecal nitrogen. The direct comparison, under identical conditions, of the digestibility of the isolated cottonseed protein with that of casein, or of some other protein known to be readily digestible, thus becomes of interest. Also, inasmuch as the toxic principle, *gossypol*, or its decomposition products (7), is always present in varying proportions in cottonseed products, it seemed that the effect of this substance upon the activity of pepsin and trypsin might prove significant.

Accordingly, three groups of experiments were made. The object of the first two was to determine the comparative final digestion *in vitro*, after the successive action of pepsin and trypsin

during definite time periods, (a) of the cottonseed globulin and casein, (b) of the pure cottonseed globulin and the cottonseed globulin to which 1 per cent of gossypol had been added, and of pure casein and casein containing 1 per cent of gossypol. The method used in these experiments was that detailed in the first paper of this series (8), with the modifications described in the second paper (9). In the third group, the time periods assigned for the action of both enzymes were somewhat extended, and a series of determinations of amino nitrogen was made and plotted in the form of a curve representing the rate of increase of the free amino nitrogen in the digests during the trypsin period.

#### EXPERIMENTAL.

The cottonseed globulin was prepared by dialysis from the aqueous sodium chloride extract of a cottonseed meal, specially treated to remove the greater part of the oil, resinous matter, and gossypol.<sup>1</sup> The preparations used for the digestion experiments were quite free from color and were readily ground to the 100 mesh powder required for the first two groups of experiments. Neither the protein nor the meal from which it was prepared had been heated in the presence of water during the processes of preparation—a point of some importance, in view of the marked increase in digestibility observed in some of the authors' previous experiments when difficultly digestible proteins were boiled for a short time with water.

The casein was prepared by precipitation with dilute hydrochloric acid, rinsed with water to remove most of the acid extracted with ether, and washed thoroughly with distilled water. In this way a preparation of very low ash content was obtained, little or no fat being present to interfere with the solution of the mineral salts by the wash water. The preparation was dehydrated with absolute alcohol, washed with ether, and dried in the usual manner. It was ground to a powder that would pass a 100 mesh sieve for the digestion tests.

The cottonseed globulin and casein preparations containing gossypol were made by dissolving the gossypol in ether, pouring

<sup>1</sup> The treatment of the meal and the method of isolating the protein will be discussed in detail in forthcoming publications.

the solution over the protein in a shallow dish, and evaporating the solvent at a temperature below 60°C. 1 per cent of gossypol was added to the protein in each case. The gossypol content of unheated cottonseed press-cake preparations cannot be very definitely stated. Experiments with whole ground cottonseed, however, indicate a yield of gossypol actually isolable from that product, amounting to from 1.5 to 5.25 per cent of the protein content.<sup>2</sup>

The first two groups of tests were made by the method already described in detail in the first (8) and second (9) papers of this series.

In the second group of experiments, 2 gm. samples were used, the volume of the reaction mixture was increased to 150 cc., and a number of samples were taken during a period of about 20 hours of tryptic digestion. Each sample was treated with 0.1 N hydrochloric acid, a few drops at a time, until thoroughly moistened. The total volume added was then made up to 110 cc., the suspension heated to 37°C., placed in a constant temperature bath at the same temperature, and treated with 40 cc. of 0.75 per cent pepsin in 0.1 N hydrochloric acid. The pepsin solution, also, was heated to 37°C., as rapidly as possible in order to minimize decomposition, and with constant agitation to avoid local overheating in the solution, before it was added to the substrate preparation. Three samples of exactly 15 cc. each were taken from each of the digests during the pepsin period.

The 105 cc. remaining after the pepsin period (4 hours) were treated with 10.5 cc. of N sodium hydroxide. A freshly prepared solution of 0.75 gm. of trypsin in 33 cc. of water was then heated quickly to 37°C., care being taken to avoid local overheating, and added to the neutralized pepsin digest. Finally, 1.5 cc. of N sodium hydroxide were added, making a total volume of 150 cc., containing 0.50 per cent of the trypsin preparation, and having a titratable alkalinity of approximately 0.01 normal. A few drops of toluene were added to prevent bacterial action. Samples were

<sup>2</sup> The figures representing the amounts of gossypol obtainable from various samples of cottonseed preparations were furnished by Dr. E. W. Schwartz, Pharmacologist in Charge, Pharmacological Laboratory, Bureau of Chemistry, who also furnished the gossypol used in this investigation.

taken at 1 hour, 2 hours, and thereafter at increasing intervals during a digestion period of about 20 hours. The samples were heated for 5 minutes in a steam bath to destroy the activity of the enzyme, and placed in cold storage until the end of the experiment. Then they were filtered and analyzed for amino nitrogen in the Van Slyke apparatus.

TABLE I.  
Comparative Digestion of Cottonseed Globulin and Casein *in Vitro*.

Preparation.	Amount of sample (pro- tein).  <i>mg.</i>	N in poly- peptide union in sample.*  <i>mg.</i>	Amino N after diges- tion (N <sub>d</sub> )  <i>mg.</i>	Amino N of blank diges- tion. (N <sub>b</sub> )  <i>mg.</i>	Diges- tion N, calcu- lated on basis of N <sub>a</sub> .  <i>per cent</i>
			(N <sub>d</sub> )  <i>mg.</i>	(N <sub>b</sub> )  <i>mg.</i>	(N <sub>a</sub> )  <i>per cent</i>
Cottonseed globulin.....	1	499.1	53.86	50.85	18.20
	2	500.6	54.02	50.85	18.20
<b>Average.....</b>					<b>60.5</b>
Casein.....	1	501.6	49.41	18.20	
	2	501.0	49.35	47.27	58.9
<b>Average.....</b>					<b>58.9</b>
Cottonseed globulin.....	3	502.1	54.18	53.92	21.27
	4	502.1	54.18	54.08	21.27
<b>Average.....</b>					<b>59.8</b>
Casein.....	3	502.0	49.45	50.99	21.27
	4	501.7	49.42	50.33	21.27
<b>Average.....</b>					<b>59.5</b>

\* Amino N after complete hydrolysis, minus the "free" amino N of the intact protein.

Curves 1 and 2, Chart 1 and Table I, show the comparative rates of digestion, under the conditions described, of the cottonseed globulin and casein. The effect of the addition of 1 per cent of its weight of gossypol upon the rate of digestion of the cottonseed globulin is shown by Curves 3 and 4, Chart 3, and in Table II. Similar experiments with casein are given in Table III. The curves are drawn to represent the *combined* effect of pepsin and

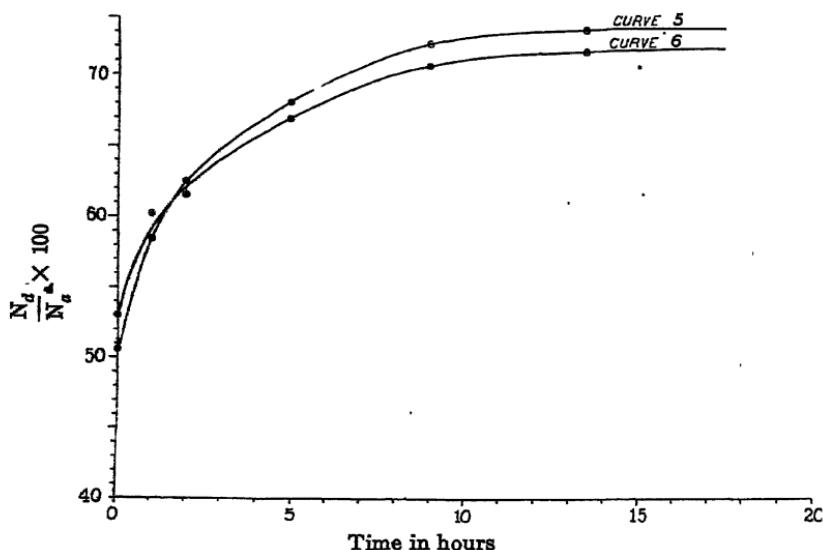
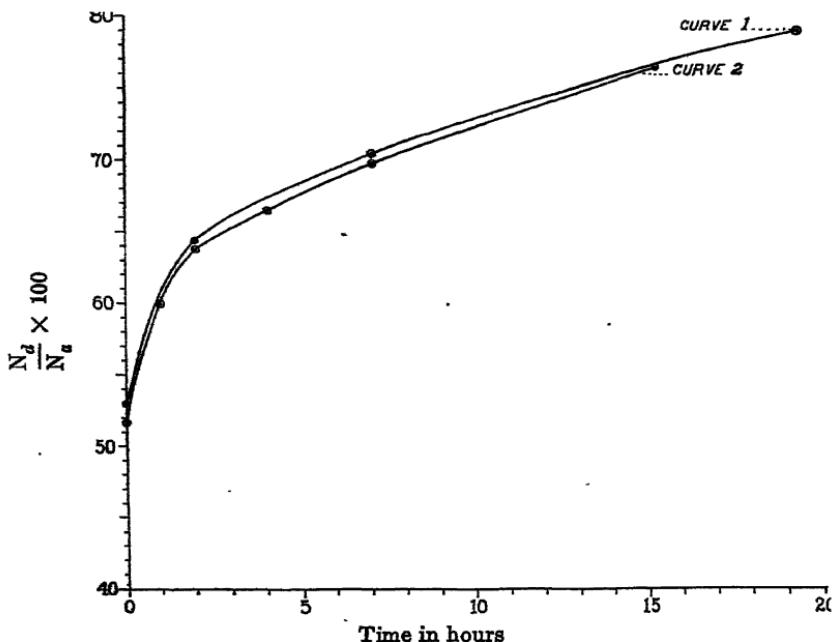
TABLE II.

*Comparative Digestion in Vitro of Pure Cottonseed Globulin and Cottonseed Globulin Containing 1 Per Cent of Gossypol.*

Preparation.	Amount of sample (protein), mg.	N in polypeptide union in sample, * (N <sub>d</sub> ) mg.	Amino N after digestion. (N <sub>d</sub> ) mg.	Amino N of blank diges- tion. (N <sub>b</sub> ) mg.	Digestion N, calculated on basis of N <sub>a</sub> . per cent
Cottonseed globulin + gossypol.....1	505.5	55.20	44.99	17.16	50.4
2	505.5	55.20	44.92	17.16	50.3
Average.....					50.4
Pure cottonseed globulin.....5	501.0	54.06	49.45	17.16	59.7
6	501.3	54.10	49.62	17.16	60.0
Average.....					59.9
Cottonseed globulin + gossypol.....3	503.1	54.94	47.75	19.03	52.3
4	503.5	54.98	47.00	19.03	50.9
Average.....					51.6
Cottonseed globulin + gossypol†.....5	505.5	55.20	44.71	19.87	45.0
6	505.4	55.19	45.05	19.87	45.6
Average.....					45.3
Pure cottonseed globulin†.....7	500.8	54.04	50.02	19.87	55.8
8	500.9	54.05	50.02	19.87	55.8
Average.....					55.8
Average of cottonseed globulin + 1 per cent of gossypol.....					51.0
"      " pure cottonseed globulin.....					60.0

\* See foot-note to Table I.

† The irregular figures of this set are not included in the averages, but are included in the table since they show the action of gossypol as well as do the more regular figures of the two preceding sets.



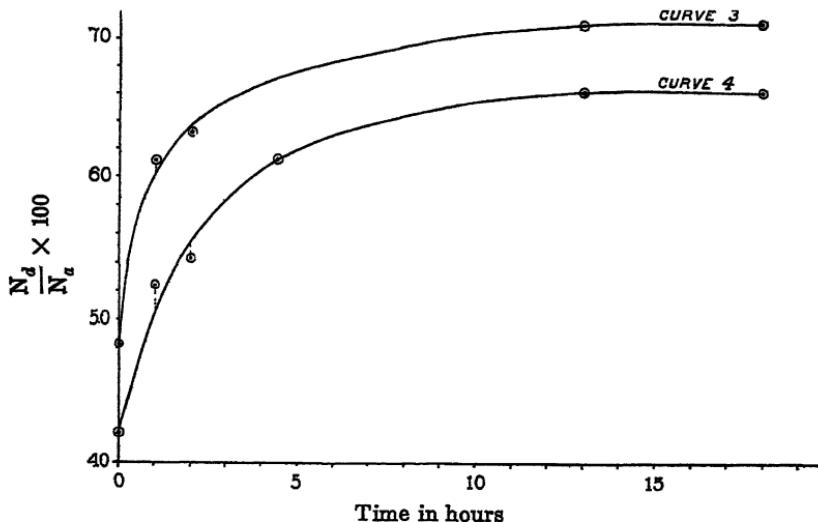


CHART 3. Comparative digestion of pure cottonseed globulin (Curve 3), and cottonseed globulin containing 1 per cent of gossypol (Curve 4). Trypsin (following pepsin) *in vitro*.

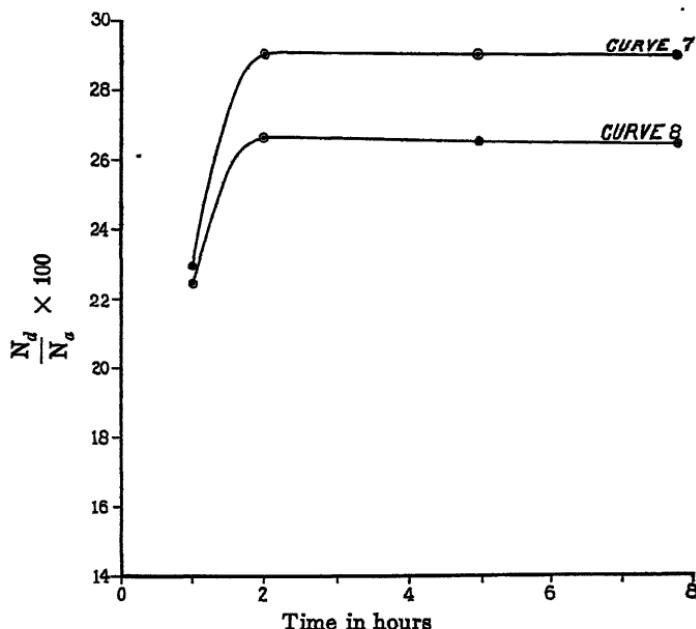


CHART 4. Comparative digestion by pepsin alone of pure cottonseed globulin (Curve 7) and cottonseed globulin containing 1 per cent of gossypol (Curve 8).

trypsin, and are started, therefore, at the termini of the pepsin curve, rather than at a common origin or zero point. The ordinates represent the quantity  $\frac{N_d}{N_a} \times 100$ , the time being plotted on the abscissæ. The blank correction,  $N_b$ , has been omitted in the calculation of the curve points on the ground that it introduces into the calculation a factor unnecessary for the purely comparative purpose of these experiments. Attention has already

TABLE III.  
*Comparative Digestion in Vitro of Casein Containing 1 Per Cent of Gossypol and Pure Casein.*

Preparation.	Amount of sample (pro- tein).	N in poly- peptide union in sample.* ( $N_a$ )	Amino N after diges- tion. ( $N_d$ )	Amino N of blank diges- tion. ( $N_b$ )	Diges- tion N, calcu- lated on basis of $N_a$ .
	mg.	mg.	mg.	mg.	per cent
Casein + gossypol.....	1	501.4	48.19	44.84	19.49
	2	501.5	48.20	44.19	19.49
Average.....					51.9
Pure casein.....	7	501.7	46.96	47.76	19.49
	8	501.6	46.95	48.09	19.49
Average.....					60.6

\* See foot-note to Table I.

been called to the fact that it is doubtful whether the omission of this blank would introduce an error greater than that resulting from the difference in the self-digestion of proteolytic enzymes in the presence and in the absence of an added substrate.<sup>3</sup> It is retained in the calculation of the results of the short period digestion, however, in order that the figures may be comparable with those given in the previous papers of the series (8 to 10).

The comparative digestion of the same preparations by pepsin alone is shown by Curves 7 and 8, Chart 4. 2 gm. samples were digested for 8 hours with a 0.5 per cent solution of pepsin preparation in 0.1 N hydrochloric acid at 37°C.

<sup>3</sup> See "Discussion," in Paper II (9).

## DISCUSSION.

In these experiments, as in our previous examinations of comparative digestibilities *in vitro*, the result is quite in accord with that of the growth experiment. In general, a protein preparation which supports a normal growth rate is readily digested by pepsin and trypsin, acting successively *in vitro* under the conditions described, giving a result closely similar to that found for casein, while no preparation found to be distinctly less readily digestible *in vitro* than casein has supported growth at a normal rate. The cottonseed globulin differs, however, from any of the vegetable proteins which have formed the subjects of the previous papers of this series, in that it produces normal growth in animals and shows a correspondingly high rate of digestion *in vitro* without any treatment other than that involved in its separation from the non-protein constituents of the seed.

The inhibitive action of gossypol (Tables II and III, and Curves 3 and 4, Chart 3, and Curves 7 and 8, Chart 4) appears sufficient to account for the incomplete digestion of the cottonseed protein fed in combination with the other constituents of cottonseed meal. For two reasons, it seems a fairly safe assumption that the isolated cottonseed globulin is almost, if not quite, 100 per cent digested by animals. In the first place, it must be very well utilized—though not necessarily perfectly, of course,—since it supports animal growth at fully the normal rate (5). In the second place, it has been shown that the cottonseed globulin is digested *in vitro*, at the same rate and to almost the same final extent (Charts 1 and 2) as casein; while the digestion of casein by animals is known to be practically complete. The digestion *in vitro* of the cottonseed globulin containing 1 per cent of gossypol is to that of the pure cottonseed globulin as 85:100 (Table II); while the digestion in animals of the protein content of cottonseed meals and flours has been found to average about 83 per cent. Such experiments *in vitro* as have been described in this paper cannot, of course, be regarded as conclusive evidence concerning the effect of gossypol upon the course of natural digestion, for the unavoidable differences between the conditions influencing the reaction *in vitro* and *in vivo* are considerable; but the fact that the addition to the isolated cottonseed globulin of

a proportion of gossypol of the same order as that found in cotton-seed meal produces *in vitro* an effect of the same order of magnitude as that observed in the natural digestion of the protein content of cottonseed meal, is certainly suggestive. Moreover, the coincidence of the above stated figures is rather striking.

The curves have been drawn to represent the combined effect of the peptic-tryptic digestion, and its progress during the trypsin period. In the case of Curves 3 and 4, representing the digestion of the cottonseed globulin alone, and in the presence of 1 per cent of its weight of gossypol, this might give the impression that gossypol is practically without effect upon trypsin, for if these curves be superimposed at their origin without change in the calculation, the gossypol cottonseed globulin curve ends at a point a little higher than the terminal point of the pure cottonseed globulin curve. If, however, the data be recalculated on the basis of the protein remaining undigested by the pepsin (the actual quantity of the substrate, so far as the trypsin *alone* is concerned) and the resulting curves drawn from zero as their origin, they show that the tryptic, as well as the peptic, digestion is affected by the gossypol. The terminal difference is less, however, in the case of trypsin alone than in the case of pepsin alone. The information of the most biological significance, however, would seem to be that represented by the figures in the tables and by the curves as they are presented; namely, that the combined effect *in vitro* of the enzymes principally concerned in natural digestion is decidedly less in the presence of gossypol.

It will be noted that no provision was made for the possible effect of the added gossypol upon the reaction of the digests. This was regarded as negligible—in fact, within the limits of manipulative variation—on account of the high molecular weight and low combining power (molecular weight, 530, with a combining power of 2 according to Carruth (7)), and the small proportion (1 part in 30,000) in which it was present in the digest. In any event it seems scarcely credible that the effect upon the reaction could be sufficient to account for the marked inhibitive effect of so small a proportion of gossypol upon peptic-tryptic digestion, or, indeed, to produce any appreciable effect.

## CONCLUSIONS.

Pepsin and trypsin, acting successfully *in vitro*, digest casein and the globulin of the cottonseed to very nearly the same extent and at practically the same rate through an extended period.

The addition to the protein of 1 per cent of its weight of the toxic principle, gossypol, known to be present in cottonseed kernels to the extent of from 1.5 to somewhat more than 5 per cent of the estimated ( $N \times 6.25$ ) protein content, interferes markedly with the digestion *in vitro* of the cottonseed globulin by pepsin and trypsin, and by pepsin alone, as well as the digestion of casein by pepsin and trypsin.

The incomplete digestion (83 per cent) by animals of the protein content of cottonseed press-cake preparations is tentatively explained as an inhibitive effect of gossypol.

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## GLUCOKININ.

### A NEW HORMONE PRESENT IN PLANT TISSUE.

#### PRELIMINARY PAPER.

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#### INTRODUCTION.

The liver of a depancreatized dog is practically glycogen-free. The respiratory quotient of such an animal is 0.70 or less. During the past year (1) the group, of which the writer was a member, working in Toronto under Professor J. J. R. Macleod, demonstrated that extracts containing the internal secretion of the pancreas in potent form, when administered to depancreatized dogs, conferred upon the livers of such animals the power of forming glycogen. The first experiment of such a nature was carried out by the writer in collaboration with Macleod, Banting, and Best (1). The analysis of the liver for glycogen in this instance gave a result of 25.6 per cent. Best and Hepburn (1) working in Macleod's laboratory, later showed that there was a marked rise in the respiratory quotient of a depancreatized dog following the administration of insulin, indicating that glucose was burning freely in the tissues of the animal. All efforts to find some *in vitro* reaction for the hormone, at this time, failed completely. The destruction of glucose or the polymerization of the same under the influence of the hormone appeared to be definitely *in vivo* phenomena.

As the power of the liver to form glycogen is so intimately associated with the presence of the pancreatic hormone in the circulation it seems obvious to predict that wherever glycogen occurs a hormone similar to, if not identical with, that produced

by the islet cells of the pancreas will be found. Two possible sources, therefore, from a purely theoretical view-point, for such a hormone would be: (1) tissues of lower animals rich in glycogen, and (2) *Saccharomyces* and other fungi, which are well known glycogen formers.

#### *Experiments with Certain Extracts of Yeast.*

Convinced that such a theory was logical and reasonable, the writer undertook to apply it. The positive results which he obtained with clam tissue (*Mya arenaria*) have already been published (2). Yeast was chosen as the working material for the next stage of the investigation, but here success did not come at once. Failure after failure resulted when yeast extracts were first used. However, with the conviction still strong that some hormone analogous to insulin must be present in yeast the investigation was continued. The first positive result was obtained on January 26, 1923, suggestive results only, having been obtained previous to this date. Since this date it has been demonstrated repeatedly that certain extracts, made either from baker's or brewer's yeast, contain a principle which is capable of producing hypoglycemia in normal rabbits. Yeast extracts which contained this principle have been prepared by five different methods and the results obtained following the administration of the same to normal animals are quite comparable.

The rabbits used were previously well fed, but during the period of observation following the injection, food was withheld and water only was available to them.

Blood sugar estimations were made by the Shaffer-Hartmann method (3).

The primary effect of the administration of the earlier extracts to normal rabbits was a great rise in the concentration of blood sugar. This effect soon passed off, however, and marked hypoglycemia followed. Also the earlier extracts were decidedly toxic in nature and the animals were extremely weak for some hours following the injection. Some died apparently as a direct result of toxic substances present in the injected solution. It was found that a good deal of the toxic action could be overcome by using relatively weak extracts. Here, however, the hypoglycemia which developed was not so striking.

The results of a number of experiments in which extracts of various types prepared from various sources were administered by subcutaneous injection to normal, well fed rabbits and the subsequent effect of the injection upon the blood sugar are shown in the form of protocols and charts.

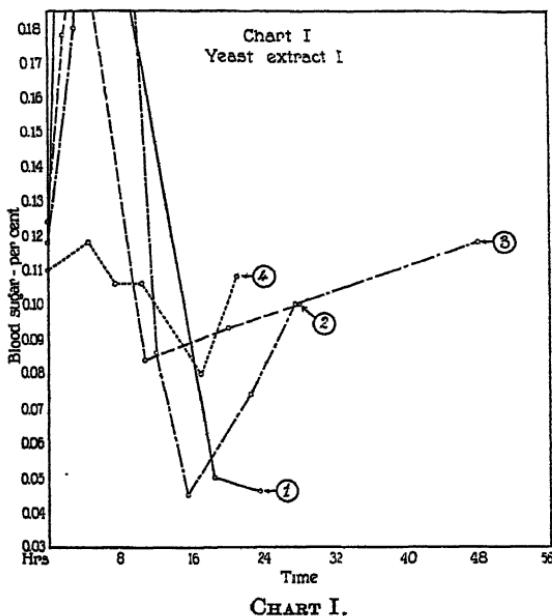


CHART I.

#### *Results of Experiments with Yeast Extract I.*

The results of four experiments in which yeast extract I was used are indicated in Chart I. It will be noticed that the low points for blood sugar were observed to occur from 10 to 24 hours following the injection of the yeast extract. This is a point of great difference from the hypoglycemia following the administration of insulin. The writer has obtained, however, "delayed action" with certain pancreatic extracts. These results have been reported elsewhere (4). Rabbit 1, Chart I, was killed 23½ hours after the injection had been made in order to estimate the glycogen content of the liver. This substance was completely lacking in that organ. Here again, however, a rabbit which has received

insulin may on occasion be found to have little or no glycogen in the liver. This experiment would have been much more convincing if the liver had contained glycogen. The blood sugar curve, Curve 2, Chart I, taken alone is not at all conclusive. The result in the case of Rabbit 3, Chart I, furnishes the most convincing evidence that the extract administered contained a potent principle. It will be noted that here there was first a marked hyperglycemia followed some hours later by a decided hypoglycemia with a subsequent return to the normal blood sugar level.

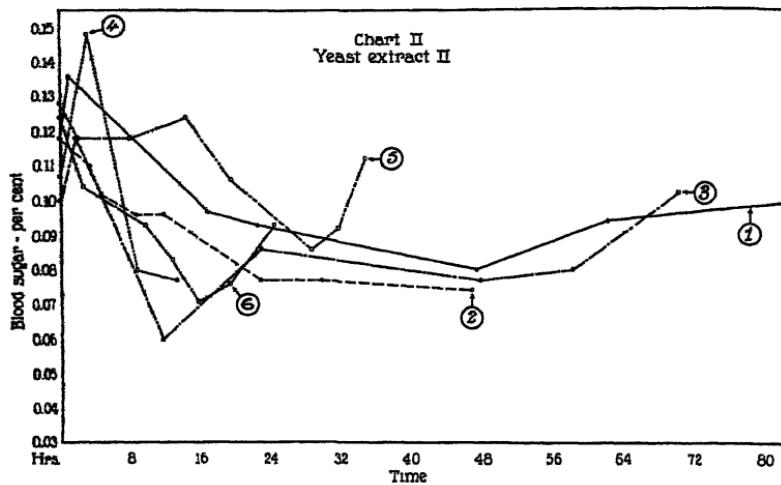


CHART II.

The result obtained in the case of Rabbit 4, Chart I, like that of Rabbit 2, Chart I, if taken alone, is not conclusive. There was no marked hyperglycemia in this instance, while at the end of 17 hours there was a slight but definite fall in the level of blood sugar.

#### *Results of Experiments with Yeast Extract II.*

Chart II illustrates some results obtained with yeast extract II. Little or no hyperglycemia was noted in this series of experiments. The experiment represented by Curve 2, Chart II, is complicated by the fact that a watery extract of fresh liver was also administered. The animal died in 47 hours. Rabbit 4 of

this series died during the night before further blood sugar determinations were made. It will be noted that there is a considerable degree of variation in these experiments between the time of injection and the time of most marked hypoglycemia.

*Results of Experiments with Yeast Extract III.*

Chart III illustrates the results of ten experiments in which yeast extract III was administered. This series of experiments furnishes absolute proof of the existence in the extracts used of a principle which produces marked hypoglycemia in normal rabbits.

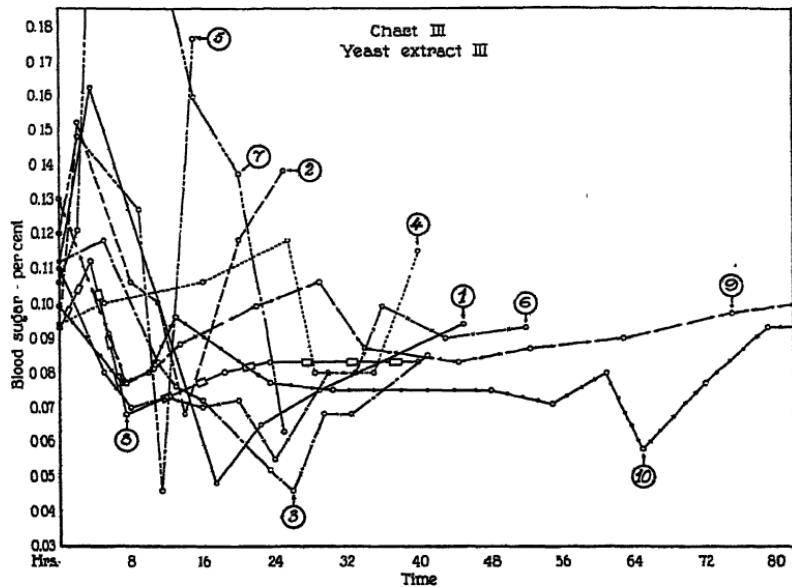


CHART III.

The occurrence of a definite hypoglycemia any time from 5 to 65 hours after the administration of the extract is a very interesting phenomenon. An unusual result was noted in the case of Rabbit 5 of this series. This animal which had been injected five times in 4 hours showed a blood sugar of 0.046 per cent, 7½ hours after the last injection, hyperglycemia then followed and the animal became very weak and died 25 hours after the first injection.

Rabbit 7 of this group was injected nine times over a period

of 20 hours. 5 hours after the last injection or 25 hours after the first, the animal had convulsions and died. The blood taken from the heart contained 0.063 per cent of glucose.

*Results of Experiments with Yeast Extract IV.*

Chart IV illustrates some results in another series of experiments with normal rabbits which were injected with yeast extract IV. Some decidedly positive results will be noted here. Rabbit 5 of this series received six injections over the first 14 hours. This

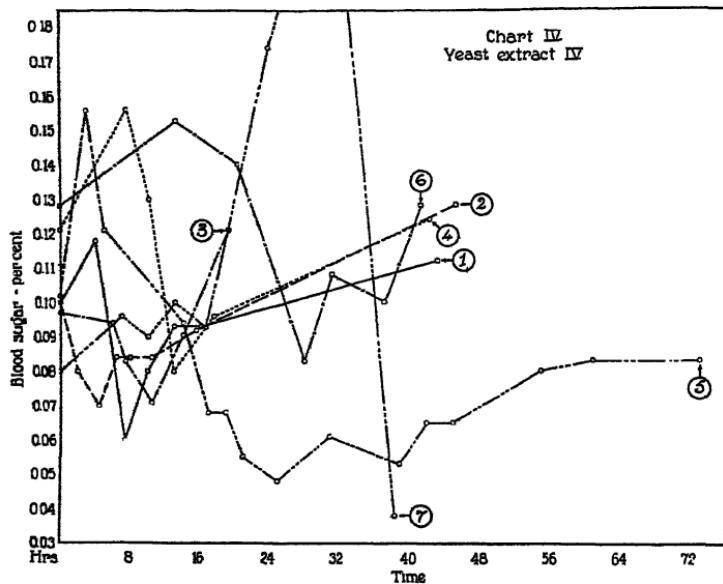


CHART IV.

resulted in a very definite hypoglycemia which slowly developed and was long maintained. Rabbit 7 received a second injection  $16\frac{1}{2}$  hours after the administration of the first dose and convulsions were noted 19 hours after the second injection. This occurred at 5 o'clock in the morning and the technician was unable to secure a blood sample. 4 hours later the animal died and a sample of blood was taken from the heart. The blood sugar concentration was then 0.038 per cent.

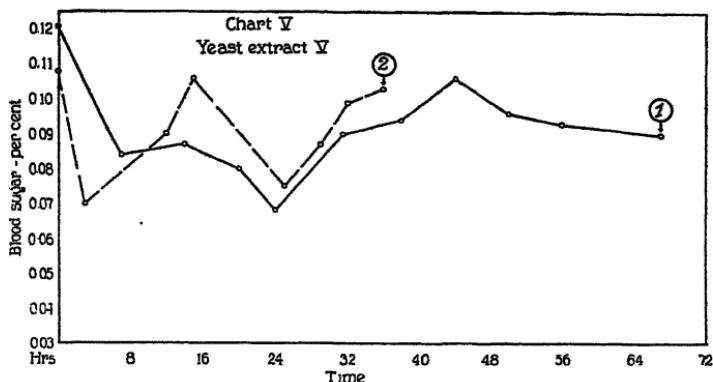


CHART V.

*Results of Experiments with Yeast Extract V.*

Two experiments in which a fifth type of extract was used are shown in Chart V.

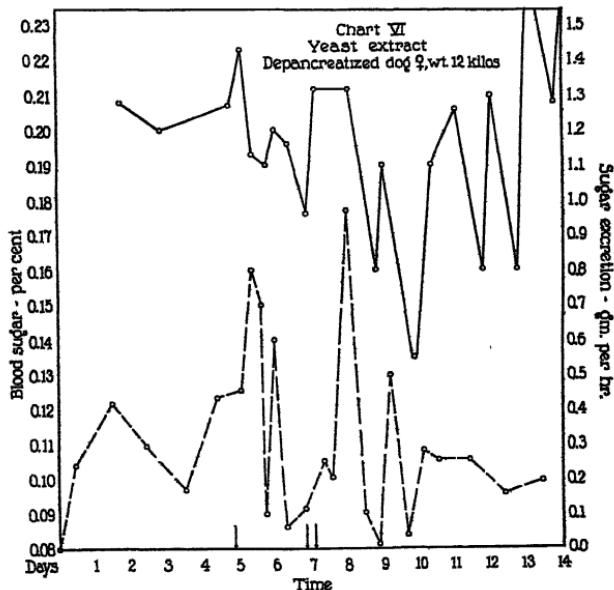


CHART VI.

*Effect of Certain Yeast Extracts upon the Depancreatized Dog.*

The blood sugar concentration and the average hourly excretion of sugar of a depancreatized dog for a period of 2 weeks is shown in Chart VI. Yeast extract was administered on the 5th day and again on the 7th day. The concentration of blood sugar reached the lowest point 0.135 per cent on the 10th day, whereas a definite decrease was observed on the 6th, 7th, and 9th days. It will be noted also that whereas the primary effect of the injection was to increase the rate of sugar excretion that this effect soon passed off and a great decrease in the rate of glucose excretion was subsequently observed, the urine being almost sugar-free for short periods on the 8th and 9th days. No toxic symptoms were observed. There is, therefore, a general parallelism between the effect of certain yeast extracts on the normal rabbit and the depancreatized dog. The animal ate ravenously the half pound of lean meat which was given to her daily throughout the experiment.

*The Evolving of a Theory.*

When such definite proof of the existence in yeast, either baker's or brewer's, of a hypoglycemia-producing principle, was obtained, the whole problem was viewed in a new light. The reasoning which was then applied was somewhat as follows: Yeast is a plant. Yeast contains a principle analogous to the internal secretion of the islet cells of the pancreas of animals. Is this principle present in yeast simply because of the glycogen-forming activity of this organism, or is the significance of this hormone in yeast much greater than would be indicated by its relationship to glycogen formation? Since Winter and Smith (5) have shown that insulin in conjunction with extract of liver can alter the optical properties of certain sugar solutions, a fact suggestive of the formation of  $\gamma$ -glucose, and since these same authors (6) have obtained results which show that the sugar of the blood of normal animals is probably  $\gamma$ -glucose, it would appear, as suggested by them, that the glucose molecule must exist in the gamma form before the liver can synthesize it or the tissues can burn it.

If, therefore, a hormone analogous to insulin is produced by the yeast cell may it not have in that organism a similar function

to that presumably performed by insulin in the animal, namely the production of  $\gamma$ -glucose, which latter when formed can either be metabolized or polymerized. If  $\gamma$ -glucose is an essential form of the molecule in the yeast plant it is also in all probability an essential form of sugar in other plants if not all plants. If this argument is logical the existence or non-existence of glycogen or even starch in a tissue (speaking now of plant tissues) would furnish no clue as to whether a hormone, the occurrence of which has been demonstrated in yeast, should or should not be present. If  $\gamma$ -glucose is essential and if its formation is dependent on a hormone, such a hormone should be found in all active tissue, or, wherever it is essential that  $\gamma$ -glucose be formed. After viewing the problem in this light the writer consulted with Professor F. J. Lewis, professor of Botany in the University of Alberta, and laid his theory before him. Dr. Lewis at once suggested the onion as a type plant with which to put the theory to the test—first, because the onion is a well recognized glucose burner; and second, because it is quite incapable of forming starch.

#### *Results of Experiments on Normal Rabbits with Onion Extracts.*

Extracts of onion tissue were therefore prepared at once and were found to have the same physiological effects as yeast extracts when administered to animals.

The first onion material used was the green tops of sprouting plants. The results of six experiments in which an injection of an extract of green onion tops was made are shown in Chart VII. Here very definite and positive results were obtained at once. These extracts also appeared to be non-toxic when administered subcutaneously, a point of much importance. The blood sugar curves represented in Chart VII are suggestive of curves in the previous charts for yeast material. That the administration of an onion extract such as this to a normal rabbit produces very definite hypoglycemia is herein established. There may be a definite fluctuation up and down in the blood sugar, as seen in Curve 3 of this series. This is a noteworthy fact.

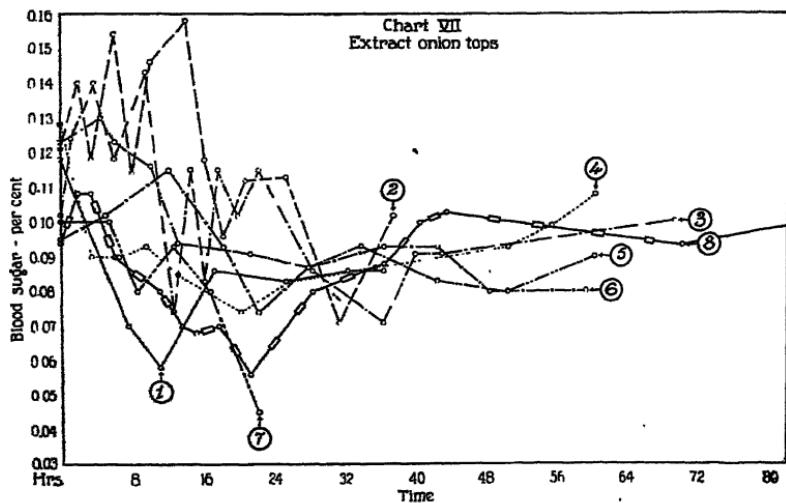
Glucokinin

CHART VII.

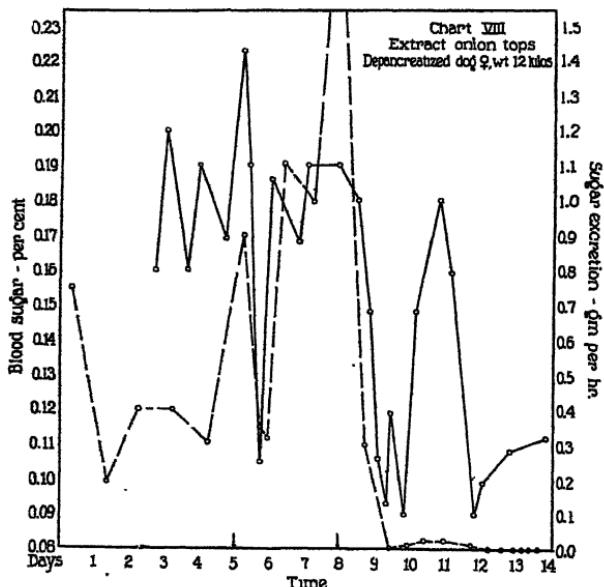


CHART VIII.

*Effect of Onion Extract upon the Depancreatized Dog.*

Chart VIII illustrates the effect of the administration of an extract made from green onion tops on the blood sugar concentration and sugar excretion of a depancreatized dog. The first injection was made 5 days after the operative procedure. No toxic effects were noted, and,  $18\frac{1}{2}$  hours later the animal had a normal blood sugar and the urine at this time was practically sugar-free. A second injection was made on the 8th day the dosage this time being increased 50 per cent. The animal's blood sugar was normal  $28\frac{1}{2}$  hours after the injection. It remained within normal limits for practically a day, rose again to a high level, only to return to normal 24 hours later, and remained normal for some days. The excretion of sugar represented in the chart in grams per hour was reduced to zero during the period of low blood sugar. This result is somewhat remarkable. It would seem doubtful as to whether the long period of normal blood sugar level and sugar-free urine was due entirely to the action of the onion extract used. These extracts were not sterile and abscess formation resulted. The possibility of the presence of a large quantity of pus being in some way associated with the long continued condition of glycosuria must not be overlooked. The presence of this hormone in bacteria is not at all unlikely. At the time of writing this animal still remains aglycosuric, is in relatively good shape, and has a ravenous appetite.

*A Survey of a Variety of Higher Plants for the New Hormone.*

Following the successful results obtained with onion extracts, various other plant tissues were tested out, and a most interesting series of positive results have been obtained. The work is as yet only in the early stages, but sufficient results have been obtained to give substantial support to the theory above propounded and to show, that, in all probability sugar metabolism to a degree at least is fundamentally the same in both the plant and the animal kingdom.

*Results with Extracts of Lettuce.*

The effect of extracts made from green lettuce leaves upon the blood sugar of normal rabbits is indicated in Chart IX. Curve 1

in this chart shows very definitely that a hypoglycemia-producing substance was present in the extract used. Slight but definite effects were also obtained by the administration of lettuce extracts to the depancreatized dog as indicated in Protocol 1, depancreatized Dog 1.

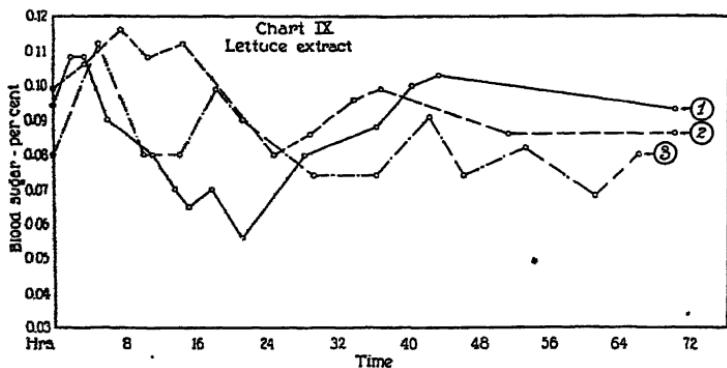


CHART IX.

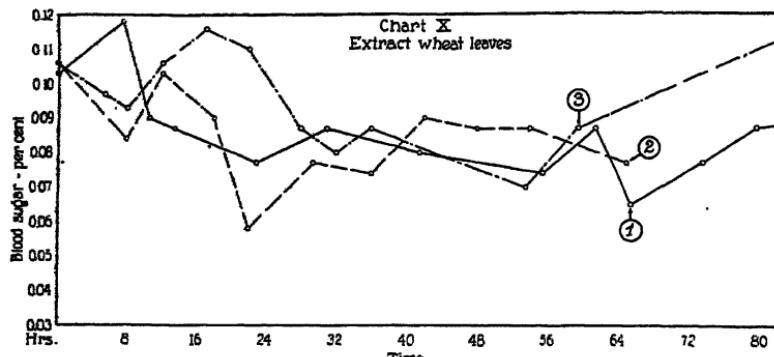


CHART X.

#### *Results with Extracts of Wheat Leaves.*

Extracts were also made from the young green leaves of sprouting wheat. Three experiments illustrating the effect of this type of extract on the normal rabbit are represented in Chart X. Here again there is definite proof of the presence of a hypoglycemia-producing principle in the injected extracts. Wheat leaves and

(probably, therefore, any young leaves of the Graminaceæ) would seem to be an excellent source for this hormone.

*Results with Extracts of a Variety of Plant Tissues.*

Chart XI represents the results of administration to normal rabbits of an extract of (1) green bean leaves and stems; (2) barley roots and sprouted grain (leaves removed); (3) onion roots and bulb; and (4) onion roots. Very definite hypoglycemia was produced in each instance. An extract of green bean leaves and

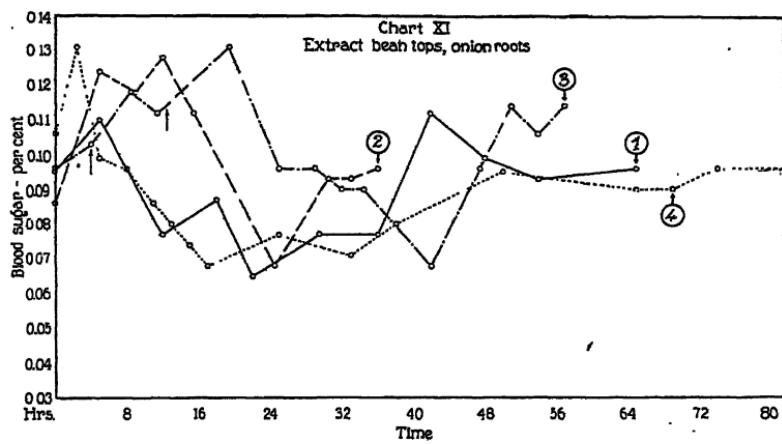


CHART XI.

stems was found to reduce the rate of sugar excretion of a depancreatized dog.

All the extracts, the physiological effects of which have been described above, were obtained from active metabolizing tissue. The results obtained with these extracts clearly indicate that a hormone associated with sugar metabolism is present in all the tissues so extracted. Whether this is true in the case of resting tissue remains to be seen. One observation made with the old leaves of *Diascia* sp. gave a negative result. Further experiments relating to this point are in progress.

The fact that many of the instances of hypoglycemia occurring after the administration of plant extracts, recorded in this paper, were noted many hours after the experiment was begun may be

criticized upon the basis that the animals were without food for many hours. Control animals have been found, however, to show a normal blood sugar even after 5 days' inanition. The lowest point observed for blood sugar concentration of starving animals was 0.080 per cent and the highest 0.130 per cent.

The discovery of this hormone in tissues of the higher plants as well as in yeast opens a new field of work in plant metabolism and affords another remarkable example of parallelism in certain physiological processes in the plant and animal kingdom. The author fully realizes that the experiments herein set forth are far from complete as yet and, therefore, not without criticism, but the results so far obtained have been so uniform in character that a preliminary paper on the work done to date seemed justified.

As the name insulin was given by the Toronto group (7) to an extract of pancreas prepared according to a definite method elaborated by the writer, this somewhat analogous hormone derived from plant sources must be known by a more general term. The name 'glucokinin' is suggested by the writer as an appropriate term, suggestive of its metabolic activity rather than its place of origin.

#### GENERAL CONCLUSION.

The fact that certain extracts made from a variety of plant tissues so divergent in character as the unicellular yeast organism, green leaves, and roots of higher plants appear to be, are capable of producing a profound disturbance in the sugar metabolism of normal rabbits and, in the few cases tested out, of depancreatized dogs, points to the existence in the plant kingdom of a hormone somewhat analogous to that produced in the higher animals by the islet cells of the pancreas. Such a hormone in all probability plays a similar rôle in the metabolism of sugar in the plant to that played by insulin in the animal. As glycogen formation is possible in the animal as a result of the activity of the pancreatic hormone it is possible that starch formation in the plant is, to a certain extent at least, dependent on the activity of glucokinin. A new field of investigation of great scope in plant physiology is hereby opened up. A few of the new problems presenting themselves are about to be studied by the author in collaboration with Dr. F. J. Lewis.

Whether this new substance will be of practical value in the treatment of diabetes mellitus remains to be shown. Extracts of a sufficient degree of purity to justify their use on human individuals have already been obtained. The actual test on the diabetic subject will be carried out shortly.

There is a decided difference between the general type of effect produced on both normal rabbits and depancreatized dogs by insulin and by glucokinin. The fall in the concentration of blood sugar in both normal and diabetic animals begins shortly after the administration of insulin. A low point is reached in from 2 to 6 hours and then the return to the original and higher level begins. To obtain the best results in the treatment of diabetic patients with insulin it is therefore necessary for the hormone to be administered frequently (two to three times daily). The effect of glucokinin, on the other hand, develops slowly and is long maintained. If it should develop, therefore, that this latter substance is serviceable in the clinic it will have a great advantage over insulin in this one respect at least. Its source would also be a point of great importance because it would be available the world over.

Owing to a degree to a lack of apparatus and to a degree to insufficient time several very important aspects of the possible activities of this substance have as yet not been studied. The effect on the respiratory quotient, on the excretion of ketone bodies, and on the alkali reserve of the diabetic subject have yet to be ascertained. A further paper will be published shortly giving more extended observations on the effect of this new hormone and details of the methods employed in the making of the extracts.

#### SUMMARY.

1. Extracts have been prepared from the vegetative tissues of several angiospermous plants as well as yeast, subcutaneous administration of which produces marked hypoglycemia in normal rabbits.
2. Certain of these extracts have been administered to depancreatized dogs. A fall in the level of blood sugar and a decrease in the hourly excretion of sugar resulted.
3. The primary effect was an increase in the rate of sugar elimination.

4. The name glucokinin has been suggested for the new hormone derived from plant sources.

5. The physiological effect of glucokinin develops slowly and is long maintained.

I wish to thank my colleague, Professor F. J. Lewis for his cooperation in suggesting likely plant tissue for testing and for supplying much of the plant material used.

This work was made possible very largely as a result of a grant to the author from the Council of the College of Physicians and Surgeons of the Province of Alberta.

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#### *Preparation of Extracts.*

*Yeast Extract I.*—Made by grinding fresh yeast with acetone (approximately 70 per cent), for several days in a ball mill, filtering, and concentrating filtrate in a warm air current. In certain instances the yeast was previously frozen with CO<sub>2</sub> snow.

*Yeast Extract II.*—The preliminary procedure was the same as in the case of yeast extract I. The concentrated acetone filtrate was purified to a degree (1) by following the method used first by the writer in preparing insulin and (2) by slightly modifying the latter process.

*Yeast Extract III.*—Fresh yeast, after dehydrating with acetone or alcohol was air-dried, pulverized, and finally extracted with hot water, temperature 70-90°C. The filtrate obtained after this extraction was used without further treatment in some instances, while in others a partial purification was effected by the use of acetone or alcohol before the extract was tested.

*Yeast Extract IV.*—Fresh yeast frozen with CO<sub>2</sub> snow was extracted at once with hot water. After filtration the extract was either used in this condition or partially purified as in the case of yeast extract III.

*Yeast Extract V.*—Fresh yeast was extracted with cold alcohol (70 to 80 per cent). The yeast was filtered off and the extract concentrated in an air current.

Extracts of plant tissues were made as follows: The fresh tissue was frozen with CO<sub>2</sub> snow. It was then pulverized by grinding in an enamel bowl. 5 volumes of 95 per cent denatured alcohol were added. After several hours the mash was filtered and the filtrate concentrated in a warm air current. In certain instances the frozen pulverized tissue was first extracted with hot water. Alcohol was then added to make about 80 per cent concentration of the reagent in the mixture. After filtration the extract was concentrated in a warm air current.

#### *Protocols of Experiments on Depancreatized Dogs.*

*Dog 1.*—Feb. 27, 3 p.m. Black retriever dog. Female. Weight 13 kilos. Depancreatized, morphine-ether anesthesia.

Feb. 28, 9.30 a.m. 240 cc. urine, 6.30 per cent sugar. 5 p.m. 200 cc. urine, 6.62 per cent sugar. Blood sugar 0.200 per cent. 7 p.m. Injection of lettuce extract. 9.30 p.m. Injection of lettuce extract. 10 p.m. 180 cc. urine, 6.26 per cent sugar.

Mar. 1, 3 a.m. 132 cc. urine, 5.54 per cent sugar. 6.30 a.m. 125 cc. urine, 3.38 per cent sugar. 9 a.m. 146 cc. urine, 2.77 per cent sugar. Blood sugar 0.194 per cent. 9.30 a.m. Injection of lettuce extract. 2 p.m. Blood sugar 0.194 per cent. 4.30 p.m. 186 cc. urine, 5.68 per cent sugar. 10 p.m. Blood sugar 0.208 per cent. 11 p.m. 270 cc. urine, 2.97 per cent sugar.

Mar. 2, 9 a.m. 260 cc. urine, 3.47 per cent sugar. 9.30 a.m. Blood sugar 0.186 per cent. Fed 8 oz. lean meat, ate ravenously. 4.15 p.m. 320 cc. urine, 3.58 per cent sugar. 4.30 p.m. Blood sugar 0.183 per cent.

Mar. 9. Animal died of peritonitis.

*Dog. 2.*—Feb. 28, 3 p.m. Black retriever dog. Female. Weight 14 kilos. Depancreatized, morphine-ether anesthesia.

• Mar. 1, 3 p.m. 342 cc. urine, 2.91 per cent sugar.

Mar. 2, 9.30 a.m. Blood sugar 0.209 per cent. 10 a.m. 375 cc. urine, 2.21 per cent sugar. 3.30 p.m. 280 cc. urine, 1.63 per cent sugar.

Mar. 3, 10 a.m. Ate 6 oz. lean meat. 1 p.m. Blood sugar 0.200 per cent. 1.30 p.m. 470 cc. urine, 2.9 per cent sugar.

Mar. 4, 11 a.m. Ate 8 oz. lean meat. 6 p.m. 456 cc. urine, 1.05 per cent sugar.

Mar. 5, 8 a.m. 216 cc. urine, 2.78 per cent sugar. 9 a.m. Blood sugar 0.206 per cent. 10 a.m. Ate 8 oz. lean meat. 12 n. Injection of yeast extract. Urine sample lost at this time. 5 p.m. Blood sugar 0.232 per cent.

Mar. 6, 12.30 a.m. Blood sugar 0.193 per cent. 200 cc. urine, 2.97 per cent sugar. 4 a.m. 90 cc. urine, 3.12 per cent sugar. 9 a.m. Blood sugar 0.190 per cent. 10 a.m. 8 oz. lean meat. 10.30 a.m. 295 cc. urine, 1.54 per cent sugar. 2 p.m. 158 cc. urine, 0.23 per cent sugar. 3.30 p.m.

Blood sugar 0.199 per cent. 11 p.m. 304 cc. urine, 1.86 per cent sugar. 11.30 p.m. Blood sugar 0.196 per cent.

Mar. 7, 9 a.m. 179 cc. urine, 0.40 per cent sugar. 10 a.m. Blood sugar 0.176 per cent. 8 oz. lean meat. 11 a.m. 151 cc. urine, 0.64 per cent sugar. 11.30 a.m. Injection of yeast extract. 5 p.m. Blood sugar 0.212 per cent. Injection of yeast extract. 8 p.m. 170 cc. urine, 1.83 per cent sugar.

Mar. 8, 5 a.m. 345 cc. urine, 0.70 per cent sugar. 10 a.m. Blood sugar 0.212 per cent. 8 oz. lean meat. 10.15 a.m. 330 cc. urine, 0.32 per cent. 4 p.m. 254 cc. urine, 1.05 per cent sugar. 9 p.m. 360 cc. urine, 2.25 per cent sugar.

Mar. 9, 9 a.m. 150 cc. urine, 0.93 per cent sugar. 10 a.m. Blood sugar 0.160 per cent. 8 oz. lean meat. 3 p.m. Blood sugar 0.170 per cent. 4 p.m. 110 cc. urine, 0.14 per cent sugar.

Mar. 10, 2 a.m. 175 cc. urine, 2.82 per cent sugar. 10 a.m. 8 oz. lean meat. 12.30 p.m. 96 cc. urine, 0.40 per cent sugar. 1 p.m. Blood sugar 0.135 per cent. 11 p.m. 180 cc. urine, 1.37 per cent sugar. 12 m. Blood sugar 0.186 per cent.

Mar. 11, 11 a.m. 8 oz. lean meat. 1 p.m. 134 cc. urine, 2.61 per cent sugar. 4 p.m. Blood sugar 0.206 per cent.

Mar. 12, 9 a.m. 164 cc. urine, 3.15 per cent sugar. 10 a.m. Blood sugar 0.160 per cent. 12 n. 8 oz. lean meat. 4.30 p.m. Blood sugar 0.230 per cent.

Mar. 13, 9 a.m. 270 cc. urine, 1.61 per cent sugar. Blood sugar 0.180 per cent. 10 a.m. 8 oz. lean meat. 4.30 p.m. Blood sugar 0.247 per cent.

Mar. 14, 9 a.m. 335 cc. urine, 1.35 per cent sugar. 10 a.m. Blood sugar 0.208 per cent. Animal at this time in fair condition.

Dog S.—Mar. 9, 3 p.m. Mongrel hound. Female. Weight 12 kilos. Depancreatized, morphine-ether anesthesia.

Mar. 10, 9 a.m. 210 cc. urine, 6.15 per cent sugar.

Mar. 11, 10 a.m. Fed 3 oz. lean meat, ate ravenously. 12 n. 320 cc. urine, 1.57 per cent sugar.

Mar. 12, 9 a.m. 216 cc. urine, 3.82 per cent sugar. 10 a.m. Blood sugar 0.160 per cent. 8 oz. lean meat. 4.30 p.m. Blood sugar 0.200 per cent.

Mar. 13, 9 a.m. 300 cc. urine, 3.18 per cent sugar. Blood sugar 0.160 per cent. 4.30 p.m. Blood sugar 0.190 per cent.

Mar. 14, 9 a.m. 235 cc. urine, 3.16 per cent sugar. 10 a.m. Blood sugar 0.168 per cent. 8 oz. lean meat. 3.30 p.m. Injection of onion top extract. 6.30 p.m. Blood sugar 0.224 per cent. 11.30 p.m. Blood sugar 0.190 per cent.

Mar. 15, 9 a.m. 415 cc. urine, 5.25 per cent sugar. 10 a.m. Blood sugar 0.106 per cent. 11 a.m. 105 cc. urine, 0.67 per cent sugar. (Note. Container not washed after last sample was taken.) 4.30 p.m. Blood sugar 0.187 per cent. 105 cc. urine, 1.05 per cent sugar.

Mar. 16, 9 a.m. 320 cc. urine, 5.95 per cent sugar. 10 a.m. Blood sugar 0.168 per cent. 8 oz. lean meat. 5 p.m. Blood sugar 0.190 per cent.

Mar. 17, 8.30 a.m. 480 cc. urine, 4.84 per cent sugar. 1 p.m. Blood sugar 0.190 per cent. 8 oz. lean meat. 1.30 p.m. 180 cc. urine, 5.74 per cent sugar. Injection of onion top extract. 4 p.m. Vomited but ate again during night. 4.30 p.m. 100 cc. urine, 8.45 per cent sugar.

Mar. 18, 1 a.m. 220 cc. urine, 7.10 per cent sugar. 3 a.m. Blood sugar 0.180 per cent. 100 cc. urine, 3.65 per cent sugar. 10 a.m. 8 oz. lean meat. 11 a.m. Blood sugar 0.148 per cent. 12.30 p.m. 190 cc. urine, 1.49 per cent sugar. 5 p.m. Blood sugar 0.106 per cent. 9.30 p.m. Blood sugar 0.093 per cent.

Mar. 19, 1 a.m. Blood sugar 0.118 per cent. 9 a.m. 108 cc. urine, 0.20 per cent sugar. 10.30 a.m. Blood sugar 0.090 per cent. 11 a.m. 6 oz. lean meat. 5 p.m. 172 cc. urine, 0.17 per cent sugar. 6 p.m. Blood sugar 0.148 per cent.

Mar. 20, 6 a.m. 208 cc. urine, 0.66 per cent sugar. 9.30 a.m. Blood sugar 0.180 per cent. 6 p.m. Blood sugar 0.158 per cent. 11 p.m. 180 cc. urine, 1.63 per cent sugar.

Mar. 21, 10 a.m. Blood sugar 0.090 per cent. Abscess opened, did not eat when fed, ate later during the night. 1.30 p.m. 146 cc. urine, 0.70 per cent sugar. 5 p.m. Blood sugar 0.099 per cent.

Mar. 22, 9 a.m. 188 cc. urine, sugar negative. 10 a.m. Blood sugar 0.108 per cent. 8 oz. lean meat.

Mar. 23, 10 a.m. Blood sugar 0.112 per cent. 435 cc. urine, sugar negative. 12 n. Large abscess opened,  $\frac{1}{2}$  pint pus removed.

Mar. 24, 9 a.m. 482 cc. urine, sugar negative. 10 a.m. 8 oz. lean meat.

## Glucokinin

*Protocols of Experiments on Normal Rabbits.*

Date.	Weight of animal.	Extract used.	Time after injection.	Blood sugar.	Remarks.
	gm.		hrs.	per cent	
Jan. 25	2,500	Yeast I.	0	0.110	
			2	0.296	
			18½	0.050	
			23½	0.046	Killed.
Jan. 28	2,000	Yeast I.	0	0.124	
			1½	0.178	
			3½	0.212	
			10½	0.084	
			20	0.093	
			28	0.100	Animal normal.
Feb. 1	2,100	Yeast I.	0	0.118	
			3	0.180	
			4½	0.390	
			6½	0.345	
			12	0.086	
			15½	0.045	
			22½	0.074	
			27½	0.100	
			48	0.118	Animal normal.
			0	0.110	
Feb. 17	2,550	Yeast I.	4½	0.118	
			7½	0.106	
			10½	0.106	
			17	0.080	
			21	0.108	
			43½	0.090	Animal normal.
			0	0.118	
			1	0.136	
Feb. 11	1,600	Yeast II.	16½	0.097	
			22	0.093	
			47	0.080	
			62	0.094	
			86	0.100	Animal normal.
			0	0.118	
			3½	0.110	
Feb. 12	2,600	Yeast II.	5½	0.102	
			8½	0.096	Water extract o. liver also injected

*Protocols of Experiments on Normal Rabbits—Continued.*

Date.	Weight of animal.	Extract used.	Time after injection.	Blood sugar.	Remarks.
	gm.		hrs.	per cent	
1923					
Feb. 12	2,600	Yeast II.	11½ 22½ 29½ 46½	0.096 0.077 0.077 0.074	Died at 52 hrs.
Feb. 13	2,000	Yeast II.	0 1½ 11½ 22½ 47½ 58 70	0.100 0.118 0.060 0.086 0.077 0.080 0.102	Animal normal.
Feb. 14	2,700	Yeast II.	0 3 8½ 13	0.107 0.148 0.080 0.077	Died during night.
Feb. 18	2,550	Yeast II.	0 2 7½ 14 19 28½ 31½ 34½ 43	0.128 0.118 0.118 0.124 0.106 0.086 0.096 0.112 0.100	Animal normal.
Feb. 19	2,500	Yeast II.	0 2½ 9½ 12½ 15½ 19 24	0.124 0.104 0.093 0.083 0.071 0.076 0.093	Animal normal.
Feb. 14	1,700	Yeast III.	0 3½ 17½ 22½ 29 45	0.112 0.162 0.048 0.065 0.075 0.094	Animal normal.

## Glucokinin

*Protocols of Experiments on Normal Rabbits—Continued.*

Date.	Weight of animal.	Extract used.	Time after injection.	Blood sugar.	Remarks.
	gm.		hrs.	per cent	
1905					
Feb. 17	1,900	Yeast III.	0 2 8 11 14 20 25	0.094 0.152 0.106 0.100 0.068 0.118 0.138	Animal very weak.
Feb. 18	1,550	Yeast III.	0 5 11 13 16 $23\frac{1}{2}$ 26 $29\frac{1}{2}$ $32\frac{1}{2}$ 41	0.112 0.118 0.083 0.076 0.072 0.052 0.046 0.068 0.068 0.085	Animal normal.
Feb. 18	2,550	Yeast III.	0 5 11 16 $25\frac{1}{2}$ $28\frac{1}{2}$ 40	0.094 0.100 0.106 0.118 0.080 0.080 0.115	Animal normal.
Feb. 21	2,930	Yeast III.	0 2 9 $11\frac{1}{2}$ 15	0.120 0.148 0.124 0.046 0.176	Two injections; 1 hr. apart. Two injections; one at 2 hrs., one at 4 hrs.
Mar. 2	2,150	Yeast III.	0 5 8 12 16 20	0.110 0.080 0.070 0.073 0.070 0.072	Died after 21 hrs.

*Protocols of Experiments on Normal Rabbits—Continued.*

Date.	Weight of animal.	Extract used.	Time after injection.	Blood sugar.	Remarks.
	gm.		hrs.	per cent	
1923					
Mar. 2	2,150	Yeast III.	24 30 33 36 43 52	0.055 0.080 0.080 0.099 0.090 0.093	Animal normal.
Mar. 1	2,900	Yeast III.	0 2½ 5 8 11 15 20 25	0.106 0.121 0.308 0.244 0.028 0.159 0.137 0.063	Nine injections during first 20 hrs.
Mar. 4	1,950	Yeast III.	0 3½ 7½ 18½ 28½ 40	0.093 0.112 0.068 0.080 0.083 0.083	Convulsions and death. Blood from heart.
Mar. 9	2,630	Yeast III.	0 5½ 7½ 10½ 13½ 22 29 34 44½ 52½ 63 77 83 87	0.130 0.091 0.077 0.081 0.088 0.099 0.106 0.087 0.083 0.087 0.090 0.097 0.100 0.087	Animal normal.
Mar. 10	2,630	Yeast III.	0 7	0.099 0.077	

## Glucokinin

*Protocols of Experiments on Normal Rabbits—Continued.*

Date.	Weight of animal.	Extract used.	Time after injection.	Blood sugar.	Remarks.
	gm.		hrs.	per cent	
1925					
Mar. 10	2,630	Yeast III.	10 13 $23\frac{1}{2}$ $30\frac{1}{2}$ 40 48 55 61 65 73 79 85 91 97 106	0.080 0.090 0.077 0.075 0.075 0.075 0.071 0.080 0.058 0.077 0.093 0.093 0.087 0.090 0.080	Animal normal.
Feb. 15	2,800	Yeast IV.	0 4 $7\frac{1}{2}$ 10 13 16 43	0.100 0.118 0.066 0.080 0.093 0.093 0.112	Animal normal.
Feb. 16	2,100	Yeast IV.	0 2 $4\frac{1}{2}$ $6\frac{1}{2}$ 8 $10\frac{1}{2}$ $15\frac{1}{2}$ 45	0.100 0.080 0.070 0.084 0.084 0.084 0.092 0.125	Animal normal.
Feb. 19	2,150	Yeast IV.	0 6 $7\frac{1}{2}$ $10\frac{1}{2}$ 14 19	0.097 0.094 0.083 0.071 0.093 0.121	Animal normal.
Feb. 21	2,500	Yeast IV.	0 $7\frac{1}{2}$	0.121 0.156	.

*Protocols of Experiments on Normal Rabbits—Continued.*

Date.	Weight of animal.	Extract used.	Time after injection: hrs.	Blood sugar. per cent	Remarks.
1923	gm.				
Feb. 21	2,500	Yeast IV.	10 13 $17\frac{1}{2}$ 42	0.130 0.080 0.096 0.124	Animal normal.
Feb. 23	2,940	Yeast IV.	0 3 5 14 17 19 21 25 31 39 42 45 61 73	0.102 0.156 0.121 0.094 0.068 0.068 0.055 0.048 0.061 0.053 0.065 0.080 0.083 0.083	Six injections during first 14 hrs.
Feb. 24	2,450	Yeast IV.	0 13 20 28 31 37 41	0.128 0.153 0.140 0.083 0.108 0.100 0.128	Animal normal.
Feb. 27	2,035	Yeast IV.	0 7 10 13 $16\frac{1}{2}$ $24\frac{1}{2}$ $31\frac{1}{2}$ $39\frac{1}{2}$	0.080 0.096 0.090 0.100 0.093 0.174 0.242 0.038	Repeated dose at 17 hrs. Convulsions at $35\frac{1}{2}$ hrs. Died.
Mar. 11	3,100	Yeast V.	0 7 14	0.121 0.084 0.087	

Glucokinin*Protocols of Experiments on Normal Rabbits—Continued.*

Date.	Weight of animal.	Extract used.	Time after injection.	Blood sugar.	Remarks.
	gm.		hrs.	per cent	
Mar. 11 1923	3,100	Yeast V.	20	0.080	
			24	0.068	
			31 $\frac{1}{2}$	0.090	
			38	0.094	
			44	0.106	
			50	0.096	
			56	0.093	
			67	0.090	Animal normal.
Mar. 18	2,400	Yeast V.	0	0.108	Fasted 2 days.
			3	0.070	
			12	0.090	
			15	0.106	
			25	0.075	
			29	0.087	
			32	0.099	
			36	0.103	Animal normal.
Feb. 20	2,950	Onion greens.	0	0.118	
			7 $\frac{1}{2}$	0.070	
			11	0.058	
			17	0.080	
			25	0.083	
			32	0.086	
			36	0.086	Animal normal.
Feb. 20	2,300	Onion greens.	0	0.121	Two injections-first 2 hrs.
			2	0.140	
			3 $\frac{1}{2}$	0.118	
			6	0.154	
			8	0.114	
			10	0.146	
			14	0.158	
			16	0.118	
			18	0.096	
			20 $\frac{1}{2}$	0.112	
			27	0.113	
			31	0.071	
			37	0.102	Animal normal.
Feb. 23	3,230	Onion greens.	0	0.102	
			1 $\frac{1}{2}$	0.124	
			3 $\frac{1}{2}$	0.140	

*Protocols of Experiments on Normal Rabbits—Continued.*

Date.	Weight of animal.	Extract used.	Time after injection.	Blood sugar.	Remarks.
1903	gm.		hrs.	percent	
Feb. 23	3,230	Onion greens.	6	0.118	
			9½	0.143	
			12½	0.074	
			14½	0.115	
			16	0.083	
			17½	0.115	
			19½	0.102	
			22	0.115	
			28	0.086	
			36	0.071	
			39½	0.091	
			42½	0.091	
			69	0.100	Animal normal.
Feb. 24	1,300	Onion greens.	0	0.128	
			3½	0.090	
			6½	0.090	
			9½	0.093	
			13	0.085	
			20	0.074	
			25½	0.083	
			35	0.087	
			50	0.093	
			55	0.099	
			60	0.108	Animal normal.
Mar.	2,480	Onion greens.	0	0.123	
			4½	0.130	
			7	0.123	
			10	0.116	
			13	0.094	
			21	0.097	
			29½	0.087	
			33½	0.083	
			42	0.083	
			50	0.080	
			60	0.090	Animal normal.
Mar.	2,475	Onion greens.	0	0.095	
			5	0.102	
			12	0.115	
			18	0.093	

## Glucokinin

*Protocols of Experiments on Normal Rabbits—Continued.*

Date.	Weight of animal. gm.	Extract used.	Time after injection. hrs.	Blood sugar. per cent	Remarks.
1923 Mar.	2,475	Onion greens.	22 29½ 36 42 48 59	0.074 0.087 0.093 0.093 0.080 0.080	Animal normal.
Mar. 22	2,035	Onion greens.	0 5½ 8½ 12½ 16½ 22	0.100 0.100 0.080 0.093 0.080 0.045	Died.
Mar. 22	1,975	Onion greens.	0 4½ 8½ 11½ 15½ 20½ 28½ 33½ 38½ 42½ 57 63	0.103 0.080 0.099 0.090 0.090 0.063 0.099 0.074 0.099 0.080 0.080 0.083	Animal normal.
Feb. 23	2,925	Lettuce.	0 2 3½ 6 11 13½ 15 17½ 21 28 36 40 43 70	0.094 0.108 0.108 0.090 0.080 0.070 0.065 0.070 0.056 0.080 0.088 0.100 0.103 0.093	Animal normal.

*Protocols of Experiments on Normal Rabbits—Continued.*

Date.	Weight of animal.	Extract used.	Time after injection.	Blood sugar.	Remarks.
	gm.		hrs.	per cent	
Feb. 28	1,530	Lettuce.	0	0.099	
			3½	0.106	
			7½	0.116	
			10½	0.108	
			14½	0.112	
			24½	0.080	
			28½	0.086	
			33½	0.096	
			36½	0.099	
			51	0.086	
Mar. 8	1,935	Lettuce.	70	0.086	Animal normal.
			0	0.080	
			5	0.112	
			10	0.080	
			14	0.080	
			18	0.099	
			21	0.090	
			29	0.074	
			36	0.074	
			42	0.091	
			46	0.074	
			53	0.082	
			61	0.068	
			66	0.080	Died 24 hrs. later.
Mar. 10	1,800	Wheat leaves.	0	0.103	
			7½	0.118	
			10½	0.090	
			13½	0.087	
			23	0.077	
			31	0.087	
			41½	0.080	
			55½	0.074	
			61½	0.087	
			65½	0.065	
			73½	0.077	
			79½	0.087	
			85½	0.106	
			91½	0.099	
			97½	0.093	
			120½	0.096	

## Glucokinin

*Protocols of Experiments on Normal Rabbits—Continued.*

Date.	Weight of animal. gm.	Extract used.	Time after injection. hrs.	Blood sugar. per cent	Remarks.
1925					
Mar. 12	2,450	Wheat leaves.	0	0.106	
			7	0.084	
			12	0.103	
			18	0.090	
			22	0.058	
			29½	0.077	
			36	0.074	
			42	0.090	
			48	0.087	
			54	0.087	
			65	0.077	Animal normal.
Mar. 23	2,080	Wheat leaves.	0	0.106	
			5½	0.097	
			8½	0.093	
			12½	0.106	
			17½	0.116	
			22½	0.110	
			28½	0.087	
			32½	0.080	
			36½	0.087	
			54	0.070	
			60	0.087	Animal normal.
Mar. 12	2,150	Bean greens.	0	0.095	
			5	0.110	
			12	0.077	
			18	0.087	
			22	0.065	
			29½	0.077	
			36	0.087	
			42	0.112	
			48	0.099	
			54	0.093	
			65	0.096	Animal normal.
Feb. 26	1,430	Onion bulb and root.	0	0.096	
			4	0.103	
			8½	0.118	
			11½	0.112	Second injection.

*Protocols of Experiments on Normal Rabbits—Concluded.*

Date.	Weight of animal.	Extract used.	Time after injection.	Blood sugar.	Remarks.
	gm.		hrs.	per cent	
1923					
Feb. 26	1,430	Onion bulb and root.	19½	0.131	Third injection at 12½ hrs.
			25	0.096	
			29	0.096	
			34½	0.090	
			42	0.068	
			47½	0.096	
			51	0.114	
			54	0.106	
			57	0.114	Animal normal.
Feb. 23	2,035	Onion greens.	0	0.106	
			2½	0.131	
			5	0.099	
			8	0.096	
			11	0.086	
			13	0.080	
			15	0.074	
			17	0.068	
			25	0.077	
			33	0.071	
			38	0.080	
			50	0.095	
			65	0.090	
			69	0.090	
			74	0.096	
			91	0.096	Animal normal.



THE SEPARATION OF THE HEXONE BASES FROM  
CERTAIN PROTEIN HYDROLYSATES BY  
ELECTROLYSIS.

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In a preliminary communication upon this subject (1) it was shown that by passing a direct current through a solution containing the hydrolytic cleavage products of gelatin which is placed in the center of a three-compartment cell, the basic amino-acids may be separated from the hydrolysate. It was further shown that when the reaction of the gelatin hydrolysate is kept at pH 7.5 arginine and lysine migrate to the cathode while histidine remains in the center compartment. If the acidity of the solution of amino-acids is adjusted to pH 5.5 the three hexone bases migrate to the cathode compartment in approximately the same proportions as they existed in the protein hydrolysate.

Since the experiments which have been reported were carried out with gelatin as the sole source for the amino-acids it appeared to us desirable to extend these investigations in order to determine the general applicability of the electrolytic method to the separation of the basic amino-acids from protein hydrolysates. Experiments were therefore carried out with the cleavage products of casein, fibrin, and red blood cells since these proteins differ markedly in their content of amino-acids.

The proteins which were used as the sources for the amino-acids were not of a high degree of purity. The casein was a commercial product such as can readily be obtained on the market. Ox blood was used as the source for fibrin. After its separation from whipped blood, the fibrin was repeatedly washed to remove hemoglobin and it was then coagulated by heat and the coagulum was washed in distilled water to remove salts. The red blood

cells were obtained by centrifuging ox blood and the product was subjected to dialysis in running water to reduce the salt content. Hydrolysis of the proteins was effected in a 30 per cent  $H_2SO_4$  solution and the acid was subsequently removed by addition of  $Ba(OH)_2$  in slight excess. The  $BaSO_4$  was filtered off and the solution was made just alkaline to phenolphthalein by further addition of  $Ba(OH)_2$  and the major portion of the ammonia was removed by blowing air through the solution.<sup>1</sup>

The electrolytic cell consisted of a rectangular wooden box  $3 \times 6 \times 4.5$  inches which was cut into three approximately equal vertical sections. For the purpose of waterproofing the surface of the cell was coated with asphalt paint. The membranes which separated the compartments consisted of strips of linen cloth which were coated with gelatin by immersion in a 30 per cent solution of this substance and the gelatin was subsequently fixed by allowing the strips to remain in formalin over night. In order to prevent leakage of fluid at the point of contact of the three portions of the cell the ends were coated with a layer of a rubber and paraffin mixture. After placing the membranes in position the three parts of the cell were clamped by means of bolts. Thin sheets of carbon  $6 \times 2$  inches were used as electrodes. During the progress of the electrolysis a small amount of carbon was found in the anode compartment and the anode gradually disintegrated; this, however, did not interfere with the electrolytic separation of the amino-acids. The solution of amino-acids which was to be subjected to electrolysis was placed in the center compartment and the reaction was maintained at the desired pH by the addition of small quantities of  $Ba(OH)_2$  solution at frequent time intervals. Similarly, the alkalinity of the cathode solution was kept at a low level by allowing a small stream of  $CO_2$  to bubble through the solution. Distilled water was placed in each of the other two compartments. Electrolysis was effected by passing about 1.5 amperes of the 110 volt direct current through the cell. The temperature was kept below  $35^{\circ}C.$  by circulating a stream of water through a test-tube which was placed in the center compartment.

<sup>1</sup> It does not appear to be essential that the ammonia be removed at this stage since its presence does not interfere with the subsequent separation of arginine and lysine by means of picrolonic acid.

and the solution was kept agitated by means of a mechanical stirrer.

The electrolysis required about 5 hours and was continued until a specimen from the center compartment, on addition of phosphotungstic acid, gave no precipitate. The electrolysis is facilitated by removing the fluid in the cathode compartment and replacing it with distilled water when the electrolysis has been about one-half completed.

TABLE I.  
*Effect of Acidity and of Reelectrolysis upon the Transport of Nitrogen to the Cathode.*

pH(approximate) of solution in center compartment.	Cathode solution.		Basic N as percentage of total N.	Remarks.
	Total N.	Total basic N.		
After electrolysis of gelatin hydrolysate.				
	mg.	mg.	percent	
6.5	980	790	81	
7.0	890	724	81	
7.5	930	740	80	
8.0	780	610	78	
After reelectrolysis of the cathode solution.				
5.5-6.0	764	760	99.5	Cathode solution from gelatin hydrolysate.
7.0-7.5	684	683	100	" " " " "
7.5-8.0	640	648	101	" " " " "
5.5-6.0	740	731	98.5	" " " casein "
7.0-7.5	652	654	100	" " " "

After the first electrolysis the cathode solution invariably contains about 20 per cent of non-basic nitrogen. This is not due to simple diffusion nor does it appear to depend on the reaction of the solution of amino-acids. The non-basic amino-acids are probably carried to the cathode by cataphoresis. On reelectrolyzing the cathode solution the amount of non-basic nitrogen was found to be reduced to an indeterminable quantity. (See Table I.) Histidine may likewise be found in the cathode after the first electrolysis, but it is reduced to a negligible quantity after reelectrolysis (see Table II).

## Separation of Hexone Bases

TABLE II.  
*Distribution of Nitrogen in the Hydrolysate from Red Blood Cells and in the Cathode Solution after Electrolysis.*

Nitrogen in original hydrolysate.	Distribution per 100 of basic nitrogen.	Nitrogen per 100 in cathode solution after reelectrolysis, pH 5.5	Yield of nitrogen which was obtained by reelectrolysis at pH 7.5 of that cathode solution which had previously been electrolyzed at pH 6.5.			Yield of nitrogen which was obtained after electrolysis at pH 7.0 - 7.5.		
			Cathode compartment, gm.	Middle compartment, per cent	Sum, per cent	Cathode compartment, gm.	Middle compartment, per cent	Sum, per cent
3.450	3.450	0.262	31	0.366	94	0.326	84	0.056
" basic nitrogen.....	".....	0.388	33	0.395	96	0	0.314	76
Arginine nitrogen.....	".....	0.414	36	0.484	94	0.410	89	0.047
Histidine "	"	0.460	36	0.484	94	0.410	89	0.047
Lysine "	"					0.457	100	0.272
						59	130	28
						0.402	87	0.402

The experimental results which were obtained are given in Tables II to V. These indicate clearly that under the conditions of the method which has been described the basic amino-acids

TABLE III.

*Distribution of Nitrogen in the Casein Hydrolysate and in the Cathode Solution after Electrolysis.\**

	Nitrogen in original hydrolysate.		Yield of nitrogen in cathode solution after reelectrolysis. pH 7.0-7.5		Yield of nitrogen in cathode solution after reelectrolysis. pH 8.5		Yield of nitrogen in cathode solution after reelectrolysis. pH 5.5	
	Nitrogen per 100 cc. of solution. gm.	Distribution of basic nitrogen. per cent	gm.	per cent	gm.	per cent	gm.	per cent
Total nitrogen.....	2.700							
" basic nitrogen.....	0.867							
Arginine nitrogen.....	0.281	32	0.260	93	0.254	90	0.260	93
Histidine ".....	0.169	20	0	0	0	0	0.155	92
Lysine ".....	0.417	48	0.362	87	0.387	93	0.364	87

\* Since the volumes of the original hydrolysates electrolyzed were not always the same the data have been unified by calculating all results on the basis of 100 cc. of original hydrolysate.

† In this and the following tables the term reelectrolysis indicates that the cathode solution which was obtained after electrolysis of the protein hydrolysate was reelectrolyzed and the analysis indicates the quantities of the basic amino-acids which were present in the cathode solution.

TABLE IV.

*Distribution of Nitrogen in the Fibrin Hydrolysate and in the Cathode Solution after Electrolysis.*

	Nitrogen in original hydrolysate.		Yield of nitrogen in cathode solution after reelectrolysis. pH 7.0-7.5	
	Nitrogen per 100 cc. of solution. gm.	Distribution of basic nitrogen. per cent	gm.	per cent
Total nitrogen.....	1.61			
" basic nitrogen.....	0.581			
Arginine nitrogen.....	0.283	.49	0.240	85
Histidine ".....	0.052	9	0	0
Lysine ".....	0.246	42	0.182	74

may be separated from the hydrolytic cleavage products (including the coloring matter) of casein, gelatin, fibrin, and red blood cells. The completeness of the separation depends on the time during which the electrolysis is continued. It has not been found profitable to electrolyze more than about 90 per cent of the hexone bases over into the cathode compartment. Arginine may be conveniently separated from lysine when they are present in the same solution by the addition of an amount of picrolonic

TABLE V.\*

*Distribution of Nitrogen in the Gelatin Hydrolysate and in the Cathode Solution after Electrolysis.*

	Nitrogen in original hydrolysate.		Yield of nitrogen in cathode solution after reelectrolysis. pH 7.5-8.0		Yield of nitrogen in cathode solution after reelectrolysis. pH 5.5-6.0	
	Nitrogen per 100 cc. of solution.	Distribution of bases to nitrogen.	gm.	per cent	gm.	per cent
Total nitrogen.....	3.050					
" basic nitrogen.....	0.925					
Arginine nitrogen.....	0.505	55	0.446	88	0.448	89
Histidine " .....	0.180	19	0	0	0.115	64
Lysine " .....	0.240	26	0.143	60	0.169	70

\* The estimation of the basic amino-acids was carried out according to the well known method of Van Slyke. It was found that the hydrolysis of arginine may conveniently be carried out by placing the flask in a boiling saturated salt solution for 6 hours. The salt solution boils at about 108° C. The ammonia was carried over into the standard acid by means of a slow current of air during the period of heating.

acid equivalent to the arginine which is present in the solution. By this procedure it was found possible to isolate (as arginine picrolonate) 80 to 85 per cent of the arginine which was present in the gelatin hydrolysate, and a yield of similar magnitude was obtained from fibrin. Our yield of lysine was not as good as would be expected. This was not due to the absence of lysine in the cathode solution, but more probably to our unfamiliarity with the method of isolating lysine as the picrate. The experi-

mental results which are shown in Table III indicate also that the electrolytic method may prove useful in the preparation of histidine. When the reaction of the fibrin hydrolysate was kept at pH 5.5 about 95 per cent of the hexone bases migrated to the cathode compartment. On reelectrolysis of this solution at pH 7.5 the major portion of the arginine and lysine was transported to the cathode, while all of the histidine together with minimum quantities of arginine and lysine remained in the center compartment. The isolation of histidine from the histidine-rich solution may be carried out with the aid of the well known technique which has been described by Fränkel (2) and by Hanke and Koessler (3).

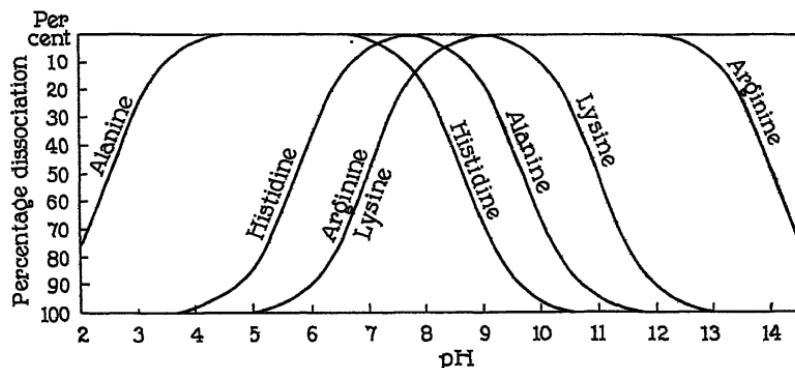


FIG. 1.

The experimental fact that when the reaction of the solution of amino-acids is kept at pH 5.5 the three basic amino-acids migrate to the cathode, while at pH 7.5 histidine remains in the center compartment and arginine and lysine migrate to the cathode is not without a theoretical foundation. In Fig. 1 we have plotted the percentage dissociation at varying pH of arginine, lysine, histidine, and alanine according to the well known method of Michaelis (4), using the dissociation constants which have been reported by Kanitz (5), Ley (6), Winkelblech (7), and Lundén (8). Since the amino-acids are amphoteric they can dissociate either as acids or as bases depending upon the reaction of the solution in which they are dissolved. At pH 7.2 the isoelectric point of histidine, the latter is only slightly dissociated while

arginine and lysine are dissociated as bases to the extent of about 40 per cent. At this reaction the latter amino-acids will migrate, under the influence of the electric current, to the cathode, while histidine, on account of being dissociated to only a slight extent, remains almost wholly in the center compartment. When the reaction of the protein hydrolysate is pH 5.5 arginine and lysine are dissociated as bases to the extent of about 95 per cent while 60 per cent of the histidine is similarly dissociated. On passing a direct current through this solution all of the basic amino-acids migrate to the cathode compartment. The dissociation curve of alanine which is representative of the majority of the amino-acids indicates that between pH 4.5 and 8.0 it is almost wholly undissociated. These amino-acids, therefore, remain in the center compartment when the electrolysis is carried out between these reaction limits. Aspartic and glutamic acids possess predominantly acid properties and between the limits of reaction at which our experiments have been carried out they should migrate to the anode compartment. This fact has been shown by Ikeda and Suzuki (9).

#### SUMMARY.

Experiments have been described which show that when the hydrolytic cleavage products of casein, gelatin, fibrin, and red blood cells are placed in the center compartment of a three-compartment cell and a direct current is passed through the solution, the basic amino-acids migrate to the cathode compartment. When the acidity of the protein hydrolysate is maintained at pH 5.5 arginine, lysine, and histidine are transported in almost the same ratio as they existed in the protein hydrolysate, while at an acidity of pH 7.5 only arginine and lysine migrate to the cathode, while histidine remains in the center compartment.

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## BASAL METABOLISM AND THE MENSTRUAL CYCLE.

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The careful work of Blunt and Dye (1) would seem at first sight to have settled negatively the question of any relationship between basal metabolism and the menstrual cycle. Their article mentions most of the literature on the subject, and calls attention to the disagreement in the results of those who have investigated the question. Zuntz (2), Gephart and Du Bois (3), and Wiltshire (4), in a more recent article, agree with Blunt and Dye that no variation of basal metabolism with the menstrual cycle can be established. Snell, Ford, and Rountree (5) state definitely that menstruation "does affect the basal metabolic rate of women at times in health and in disease," and promise publication of details, which, as far as the present writer can ascertain, have not yet appeared.

The work of Zuntz was done on two subjects only. Ford studied ten cases, but his data are not available for critical examination. Gephart and Du Bois give the subject merely passing mention. Miss Wiltshire made a large number of determinations on five subjects, but publishes only her average results. The data of Blunt and Dye are given with admirable completeness with the single exception that in a number of cases the dates of the preceding or following menstrual periods are lacking, making it impossible to refer these determinations to the exact time of menstruation.

The last mentioned authors, together with Miss Wiltshire, divide the menstrual cycle into four phases, *viz.* premenstrual, menstrual, postmenstrual, and intermenstrual, and endeavor, by comparing the averages for these four periods, to ascertain whether menstruation affects basal metabolism. Their conclusions, as stated above, are negative. Miss Wiltshire states: "The variations during the different phases of the sexual cycle are so small that they cannot be regarded as showing any marked effect due to menstruation;" and further, that "the fluctuations which normally occur are often greater than these variations," leaving the subject at that point. It is the object of this paper to report and analyze data less extensive, indeed, than those of the above mentioned authors, but obtained under circumstances in some respects more favorable, and then, by submitting the fundamental data of Blunt and Dye to the analytical methods used in this article, to show that certain quite definite positive inferences can be deduced from them. The fact that normal daily variations of basal metabolism exceed any which may be due to the menstrual cycle does not justify the assumption that the latter are unimportant or undemonstrable.

TABLE I.  
*Fundamental Data.*

Subject No.	Age.	Date of experiment.	Date of last menstrual period.	Weight of subject.	Height of subject.	O <sub>2</sub> consumption per min.	Normal O <sub>2</sub> consumption per min.	Percentage above or below normal.
	yr.			lbs.	in.	cc.	cc.	per cent
1	22	Sept. 23	Aug. 22	118	59	191	177	+8
		Oct. 1	Sept. 24	120	59	190	179	+6
		" 8	Oct. 8	121	59	202	180	+12
		" 15	" 8	124	59	184	182	+1
		" 28	" 8	122	59	192	181	+6
2	24	Sept. 24	Sept. 16	128	69	230	206	+11
		Oct. 10	" 16	131	69	252	208	+21
		" 20	Oct. 16	135	69	236	211	+12
		" 27	" 16	136	69	224	212	+6
3	20	Sept. 26	Sept. 18	114	65	188	186	+1
		Oct. 10	" 18	114	65	200	186	+8
		" 15	" 18	115	65	215	187	+15
		" 25	Oct. 16	118	65	210	188	+11
		" 29	" 16	114	65	203	186	+9
		Nov. 8	" 16	115	65	180	186	-3
			Nov. 23					
4	24	Sept. 26	Sept. 18	134	61	225	192	+17
		Oct. 9	" 18	131	61	251	190	+30
		" 15	Oct. 10	133	61	252	191	+30
		" 24	" 10	134	61	250	192	+29
5	23	Sept. 28	Sept. 8	145	69	220	217	+1
		Oct. 11	" 8	145	69	210	217	-3
		" 29	Oct. 16	132	69	254	212	+20
		Nov. 4	Nov. 2	133	69	230	212	+8
6	21	Sept. 30	Sept. 16	139	63	208	203	+2
		Oct. 15	" 16	140	63	202	204	-1
			Oct. 24					
7	20	Sept. 30	Sept. 2	115	64	224	188	+19
		Oct. 9	Oct. 4	112	64	201	186	+8
		" 11	" 4	116	64	198	189	+6
		" 24	" 4	115	64	202	188	+7
8	26	Oct. 2	Sept. 24	121	64	204	187	+9
		" 16	" 24	124	64	219	189	+16
			Oct. 22					
9	28	Oct. 2	" 5	112	65	224	185	+20
		" 12	" 4	115	65	199	188	+6
10	22	" 6	Sept. 11	156	65	252	213	+18
		" 12	Oct. 8	160	65	232	217	+7

TABLE I—Continued.

Subject No.	Age.	Date of experiment.	Date of last menstrual period.	Weight of subject.	Height of subject.	O <sub>2</sub> consumption per min.	Normal O <sub>2</sub> consumption per min.	Percentage above or below normal.
	Yrs.			lbs.	in.	cc.	cc.	per cent
11	29	Oct. 8	Sept. 16	142	64	241	204	+18
		" 13	" 16	145	64	257	205	+26
		" 22	Oct. 14	146	64	220	206	+7
12	21	" 5	" 1	111	64	188	183	+3
		" 16	" 1	114	64	182	184	-1
		" 20	" 1	115	64	187	185	+1
13	27	" 5	" 1	118	66	184	189	-3
		" 17	" 1	121	66	178	191	-6
		" 25	" 1	119	66	200	190	+5
		" 28	" 1	120	66	204	191	+7
		Nov. 3	" 30	118	66	195	189	+3
		" 10	" 30	120	66	190	191	+0
		Oct. 6	" 2	141	61	203	195	+4
14	19	" 18	" 2	144	61	195	198	-2
		" 26	" 2	145	61	220	199	+10
		Nov. 6	Nov. 4	143	61	212	197	+7
15	20	Oct. 8	Sept. 29	126	66	215	200	+7
		" 19	" 29	124	66	223	196	+13
		" 26	" 29	121	66	227	196	+16
			Oct. 28					
16	35	Nov. 19	" 25	145	63	185	199	-7
		" 22	" 25	148	63	190	202	-6
		" 29	Nov. 23	146	63	180	200	-10
17	26	" 19	" 1	93	63	162	167	-3
		" 30	" 1	92	63	160	165	-3
		Dec. 12	" 1	90	63	150	162	-7
		" 18	Dec. 18	92	63	158	166	-5
		" 23	" 18	94	63	154	168	-9
18	32	Nov. 20	Nov. 10	188	66	236	230	+3
		" 30	" 10	190	66	250	231	+8
19	20	" 21	Oct. 28	114	64	191	185	+3
		" 24	" 28	117	64	184	186	-1
		Dec. 1	" 28	117	64	203	186	+9
		" 8	Dec. 3	117	64	190	186	+2
		" 13	" 3	118	64	177	186	-5
20	21	Nov. 21	Nov. 6	145	65	178	206	-14
		" 24	" 6	142	65	193	205	-6
		Dec. 1	" 30	142	65	196	205	-4
		" 8	" 30	143	65	187	206	-10
		" 13	" 30	143	65	188	206	-9

TABLE I—Concluded.

Subject No.	Age.	Date of experiment.	Date of last menstrual period.	Weight of subject.	Height of subject.	O <sub>2</sub> consumption per min.	Normal O <sub>2</sub> consumption per min.	Percentage above or below normal.
	yrs.			lbs.	in.	cc.	cc.	per cent
21	22	Nov. 22	Nov. 21	123	63	186	190	-2
		" 26	" 21	124	63	197	190	+4
		Dec. 3	" 21	122	63	192	189	+2
		" 10	" 21	121	63	211	187	+13
		" 18	Dec. 16	122	63	171	188	-9
		" 21	" 16	122	63	177	189	-6
22	19	Nov. 22	Nov. 10	134	62	188	193	-3
		" 26	" 10	134	62	195	193	+1
		Dec. 3	" 10	136	62	222	194	+14
		" 10	Dec. 4	135	62	191	193	-1
		" 17	" 4	135	62	189	193	-2
		" 21	" 4	136	62	197	194	+2
23	18	Nov. 23	Nov. 18	110	62	176	179	-2
		" 30	" 18	110	62	175	179	-2
		Dec. 7	" 18	111	62	165	179	-8
		" 11	" 18	112	62	140	180	-22
		" 19	" 18	110	62	142	179	-20
		" 24	" 18	109	62	162	178	-8
24	20		Jan. 3					
		Nov. 23	Oct. 30	122	64	204	191	+7
		" 30	Nov. 29	121	64	200	189	+6
		Dec. 7	" 29	123	64	188	192	-2
		" 11	" 29	123	64	199	192	+3
		" 19	" 29	126	64	190	194	-2
		" 24	" 29	127	64	202	195	+4
			Dec. 27					

## EXPERIMENTAL.

The fundamental data of Table I give the results of ninety-eight basal metabolism determinations on twenty-four subjects, under conditions of uniformity not often obtainable in such work. Twenty of the subjects were nurses in training. Their mode of life and daily routine were subject to but little variation. Their meals were all taken at the same hours in the same vegetarian café, conducted by the institution in which they were employed, and the food at their disposal was almost monotonously uniform. All the determinations were made on the same apparatus (San-

born-Benedict) by the writer and an assistant technician, at the same hour of the day (between 6 and 7 a.m.). The subjects were taken in pairs for the most part, and slept the preceding night in the metabolism laboratory. A light vegetarian supper at 5 p.m. was in every case the last preceding meal, so their condition was postabsorptive. The determinations were made before the subject moved in the morning, and the net weight, pulse, and temperature (if there was any indication of fever) were at once observed. Pulse and temperature are not recorded, as they were

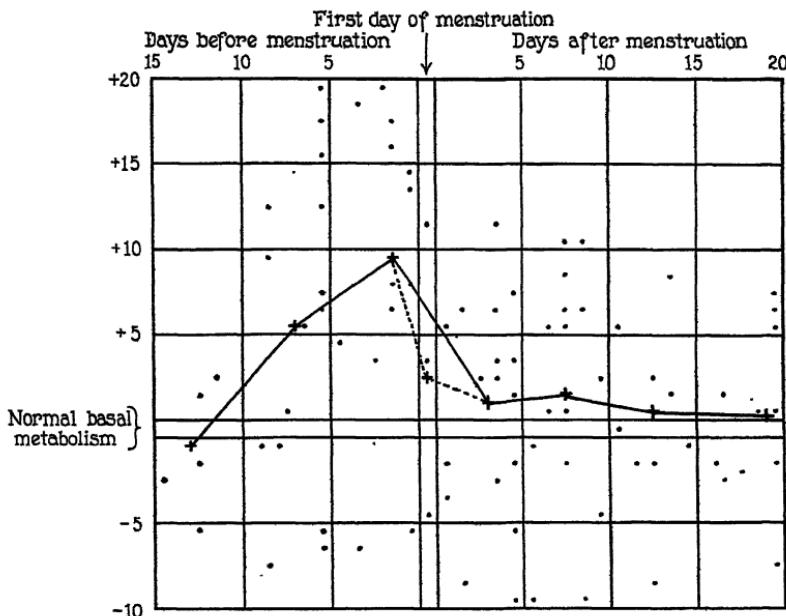


CHART 1.

within normal limits in all cases the data of which are subjected to analysis. Most of the subjects were in vigorous health, engaged in heavy manual labor from 7 to 12 hours daily, and sleeping soundly at night.

The oxygen consumption was estimated for two or three 10 minute periods, or until agreement within 3 per cent was reached. As convenient arbitrary "normal" basal metabolism values the tables given in Sanborn's "Basal metabolism" (6) were used.

These are based on (a) the well known body surface formula of Du Bois, (b) the table of calories given by Aub and Du Bois (7) from which values (c), at the suggestion of Aub and Means (8), 1.8 calories are subtracted. The validity of this "normal" is not considered in the present paper. All individual results are given to the nearest whole numbers.

Chart 1 represents graphically the percentages in the last column of Table I. The data are plotted horizontally with regard to the 1st day of the nearest menstrual period, and verti-

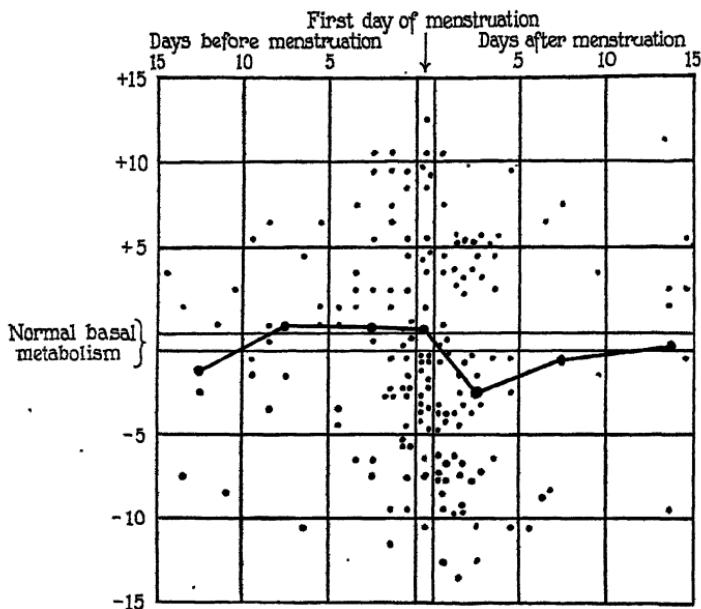


CHART 2.

cially with reference to the "normal" as defined above. A graph is drawn connecting the means of successive 5 day periods, the dotted line passing through the mean of the three determinations made on the 1st day of menstruation. The total number of observations is, indeed, small, representing about the minimum to which such a method could be applied. But the considerable daily fluctuations observed in basal metabolism, even when every possible precaution to secure uniformity of conditions is taken,

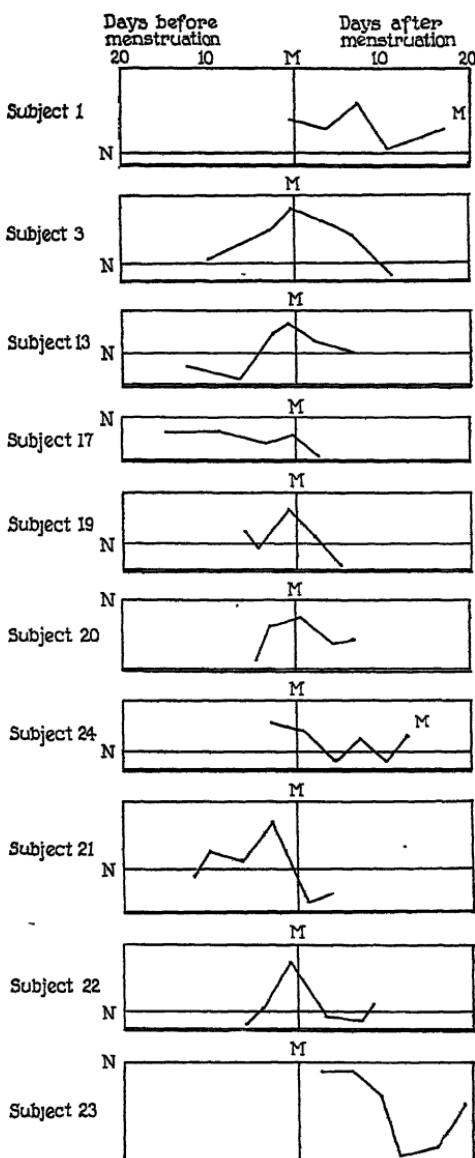


CHART 3.

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easily masks, in any individual case, smaller variations due to some regular function like menstruation, and make it necessary to submit considerable data to some kind of statistical analysis if these smaller variations are to be detected.

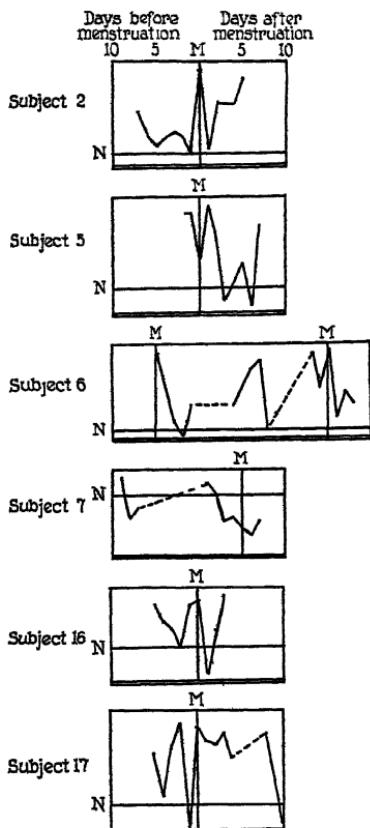


CHART 4. Compiled from data of Blunt and Dye.

Chart 2 subjects to the same method of analysis such of the data of Blunt and Dye as could be oriented with reference to the 1st day of the menstrual period. None of their determinations which could be thus placed is omitted from the chart. As quite a number of determinations fell on the 1st days of various menstrual periods, the mean of the values for this day is taken as one point on the graph.

In Chart 3, graphs representing the basal metabolic fluctuations of a number of individual cases are given, the horizontal lines "N" being the "normals" as defined above, and the vertical lines "M" representing the 1st day of the menstrual period occurring during the course of the experiment. Chart 4 represents some of Blunt and Dye's data in the same way.

#### *Notes and Observations on the Table and Charts.*

All the determinations of basal metabolism on all the subjects chosen for this investigation are recorded in Table I, including some which are obviously abnormal.

Subject 1 presents a menstrual cycle of 20 days, which her history showed to have persisted since puberty.

Subject 4, a young married woman, without children, of sedentary occupation, apparently in good health, gave abnormally high results for no reason which could be assigned. The data in this case are not used in Chart 1.

Subject 5 was severely burned on Oct. 28, and the data of determinations made after the accident are not used in Chart 1, as there was some fever.

Subjects 13 and 17 were engaged in sedentary work (typists).

Subjects 1 to 12 were all nurses approaching the completion of a 3 year training period. They were girls of exceptional strength, survivors of a class originally three times as large, and were working at high pressure, preparing for their graduation exercises as well as performing their regular duties. Their average basal metabolism was 10.1 per cent above the arbitrary "normal" used in these experiments.

Subjects 19 to 24, on the other hand, were younger girls, just beginning their course of training. The determinations were made after the rush season of the institution was over, while the girls were attending two or three classes daily, and not doing, on the average, more than 7 hours of daily manual labor. Their average basal metabolism was 2.1 per cent below the arbitrary normal, which agrees very well with the results obtained by Blunt and Dye for women engaged in sedentary occupations.

In the construction of Chart 1, the following data from Table I were omitted: (a) Data of a clearly abnormal character as noted above; (b) all determinations which were more than 20 per cent above, or 10 per cent below, the arbitrary "normal." These limits were chosen (a) because nearly all of the data lay between them and (b) because the average of all the determinations made was nearly 5 per cent above the arbitrary "normal."

The rise in the graph representing the average basal metabolism in all these cases preceding menstruation, followed by a fall equally pronounced, is so striking that the writer was inclined to look for some factor of error, and did not feel like trusting his own results until a similar analysis of the data of Blunt and Dye confirmed, in part at least, these observations.

All the data of Blunt and Dye which could be placed with reference to the 1st day of menstruation are used in Chart 2. The graph of average basal metabolism determinations shows a small but distinct rise during the period between 7 and 15 days before menstruation. More significant is the sharp fall immediately after the 1st day of menstruation. The large number of determinations made just before and during the 1st week of menstruation gives this result great weight as evidence of the generality of this phenomenon.

Charts 3 and 4 give graphs of individual fluctuations in basal metabolism of some of the subjects of the above experiments. The graphs of Chart 3, from the writer's subjects, are not of great significance, taken individually, because of the small number of determinations made in each case. And even the graphs of Chart 4, taken from Blunt and Dye's data, while much more complete, serve chiefly to demonstrate that individual daily fluctuations are so much greater than the periodic monthly variation that the latter can be demonstrated only by statistical methods.

#### DISCUSSION.

Some of the above observations would be valueless standing by themselves, but the fact that they all point in the same direction can hardly be without significance. In spite of the great irregularity and apparent confusion of the data, one feature stands out clearly, and that is the distinct fall in basal metabolism about the beginning of the menstrual period. This is confirmed by every method of analysis used for both sets of data, and further emphasized by the following:

*Comparison of Data of Blunt and Dye, and Wakeham.*

	Blunt and Dye.	Wake- ham.
	per cent	per cent
Average of all premenstrual determinations (within 15 days of 1st day of menstruation).....	+0.5	+5.7
Average of all postmenstrual determinations (within 15 days after menstruation, omitting 1st day).....	-1.5	+1.7
Difference between premenstrual and postmenstrual phases (being in each case a decrease).....	2.0	4.0
Number of premenstrual determinations above normal.....	37	30
"    "    "    "    below    "    .....	29	13
"    "    postmenstrual    "    above    "    .....	28	31
"    "    "    "    below    "    .....	46	23
Percentage of total premenstrual "    above    "    .....	56	70
"    "    postmenstrual    "    "    "    .....	37	57.4
Drop in these percentages due (presumably) to menstruation.....	19	12.6

The writer's data indicate a distinct rise in basal metabolism within the week or 10 days prior to menstruation. This is not so clearly shown by Blunt and Dye's results. But if there is a fall after menstruation, there must be a rise at some phase of the menstrual cycle. Blunt and Dye made very few determinations during the 2nd and 3rd weeks after menstruation, and the effect of other factors disturbing basal metabolism seems to have masked the influence of the menstrual cycle. The writer's data, obtained from subjects whose mode of life and general habits were presumably much more uniform than those of Blunt and Dye's subjects, indicate a premenstrual rise beginning a week or 10 days before menstruation, a sudden drop at the beginning of the menstrual period (this is confirmed by Blunt and Dye's data) followed by a gradual return to normality within from 7 to 10 days.

The analysis shows that whatever variation there is due to the menstrual cycle must be much smaller than the daily fluctuations caused by other factors. It is therefore to the point to emphasize the necessity of obtaining, for investigation of this question, not only as large a number of data as possible, but also the greatest possible uniformity of conditions. For analytical purposes it would also be desirable to have determinations evenly distributed throughout the menstrual cycle.

Further possible inferences from comparison of the two sets of data used in this article are that the average basal metabolism of women engaged in strenuous physical labor is higher, perhaps by 10 per cent, than of those in sedentary occupations, that basal metabolism fluctuations due to the menstrual cycle are at least twice as great in the former as in the latter class, and that, in normal subjects, uniform habits of life are accompanied by a smaller range of individual daily fluctuation than occurs in individuals who lead a more varied existence. This last point is made clear in the following tabulation, in which only presumably normal subjects are considered:

	Blunt and Dye.	Wakeham.
	per cent	per cent
Maximum daily variation.....	28.8	20
Minimum    "        " .....	7.4	6
Average    "        " .....	13.2	12.4

*Results.*

It is shown by the analysis of data given by the writer, and others by Blunt and Dye (who did not think that their data revealed any connection between basal metabolism and the menstrual cycle) that there is a distinct fall in basal metabolism during or immediately after menstruation.

The writer's data indicate a premenstrual rise in basal metabolism, but this conclusion is not deducible from the data of Blunt and Dye.

Further suggestions, which must be regarded as merely tentative are:

1. That basal metabolism is considerably higher, on the average, in those engaged in strenuous labor than in those of sedentary occupations.
2. That basal metabolism fluctuations in those living under uniform conditions are less than in those whose mode of life is more varied.
3. That the basal metabolism variation due to the menstrual cycle, while in every case less than the average daily fluctuations, is greater in active individuals than in sedentary individuals.
4. That causes which produce too frequent menstruation are likely to be accompanied by high basal metabolism.
5. That causes which delay or suppress menstruation are likely to be accompanied by low basal metabolism.

The writer begs leave, in conclusion, to emphasize the impossibility of considering any of these results final except, perhaps, the first one, and to hope that this article will lead to the publication of further data which may enable these questions to be satisfactorily settled.

My best thanks are due to the nurses who, at considerable personal inconvenience, cooperated in this investigation; to the assistants who carried out much of the work here recorded; and, most of all, to Dr. Robert C. Lewis, Director of the Denison Research Laboratory and Professor of Biochemistry, University of Colorado, for his helpful suggestions and criticism in preparing this paper for publication.

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## THE ACID-BASE EQUILIBRIUM IN SIMPLE TWO-PHASE SYSTEMS.

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The recognition of the association of the acid-base equilibrium with many, if not all, physiological processes has led many investigators of biological phenomena to study relationships between this equilibrium and particular systems in which they were interested. Such relationships are usually expressed in terms of the hydrogen ion concentration, the value of which enables one to calculate the ratio of the concentration of any free weak acid (or base) to the concentration of its salt. This applies, within ordinary limits, to a solution of a mixture of several acids and their salts, each acid being considered independently of the others. Many problems, for example the states in which  $\text{CO}_2$  exists in the blood, are, for all practical purposes, solved by this method.

The subject of this paper deals with a simple application of the method, together with the application of the distribution law, to two-phase systems at equilibrium. Many general and specific investigations of the acid-base equilibrium and its relation to the physicochemical behavior of proteins have given us a rational foundation for the description of these systems, and Loeb (1) has recently combined these foundations with the Donnan theory to interpret the colloidal properties of proteins. Considering the advance that has accompanied these researches, it is a curious fact that no similar general studies of the relationship between the acid-base equilibrium and lipoids have become widely known, if they have been attempted at all. That is to say, we are not aware of any treatment of the general equilibrium existing in the system: fat (or fat solvent), fatty acid, salt, base, water.

This fact, and the desirability of an examination of this equilibrium, were mentioned to me by Professor L. J. Henderson, whom I particularly wish to thank for these suggestions, as well as for many opportunities he has afforded me in the past for studying the possibilities involved in the method of analysis to be used.

The general problem to be discussed in this paper deals with the distribution of a weak acid between an aqueous solution at various hydrogen ion concentrations and any other simple phase (in which the acid may exist) in equilibrium with this solution. Distribution, in this sense, refers to all the forms in which the acid may exist; *i.e.*, aggregated, dissociated, as salt, etc. For example, lactic acid distributes itself between amyl alcohol and water. On the other hand, Na lactate would be present almost entirely in the aqueous phase. Obviously, then, if NaOH is added to the system, lactic acid will migrate from the alcohol into the water following the formation of Na lactate in the aqueous phase. The problem is to relate the total distribution of acid with the hydrogen ion concentration, and to find out any other derived relations which may be of interest.

#### THEORETICAL.

It will be convenient to make clear certain crucial points involved in the two generalizations we shall use; namely, the mass law and the distribution law as they apply to the problem.

Henderson (2) derived the following equation from the mass law:

$$[H] = \frac{[HA]}{[BA]} \times \frac{K}{\gamma}$$

and this equation has been repeatedly verified by experiment (see Clark's book (3)). Now in these experiments the apparent and obvious values for HA and BA, when substituted in the equation, lead to consistent results; and this fact leads to the conclusion that the addition of the salt of a weak acid to a solution containing the weak acid, does not effect any change in the concentration of the free undissociated acid. In other words, addition of BA results in a corresponding change in H, but no change in HA. Conversely, addition of HA results in a change

in H but not in BA. This statement is evidently true as a first approximation, in fact any deviation from this rule would certainly be very small compared to the magnitude of the changes the equation is designed to express, especially with weak acids, and in the range of usefulness of the equation. Evident as this may seem, it is emphasized because it leads to an apparent contradiction of a statement made by Nernst to which reference will be made later.

The distribution law (4) involves the proposition that the distribution ratio of any given molecular species between two phases is a constant independent of the presence of any other species except as these may modify the thermodynamic environment of the respective phases.

These two well known generalizations lead to the equations developed below. The calculations consist merely in taking a known amount of a weak acid, some of which exists as salt. The remainder, free undissociated acid, is distributed between two phases according to a known ratio. The concentration of free acid remaining in the aqueous phase divided by the concentration of its salt leads to a value for the hydrogen ion concentration. The salt formed is assumed to be present in the aqueous phase only. The solutions are supposed to be sufficiently dilute to come within the range of applicability of the mass law, and the volumes of the two phases are assumed to remain constant (unless otherwise specified), and the temperature is assumed to be constant. The calculations are so simple that we would hardly feel justified in presenting them were it not for the fact that the consequences involved in the equations developed are not directly obvious from the original assumptions and appear to be of some interest. Moreover, the general laws of physics and chemistry become increasingly important to the physiologist as they are applied more and more specifically to those special conditions which characterize the organism.

Three different cases, to be designated A, B, and C, will be treated, differing from each other in respect to the nature of the existence of the weak acid in the non-aqueous phase. The following notation will be used:

$M$  = total amount of weak acid, in gram molecules, present in any form of combination in the system.

$V_w$  = volume of aqueous phase, in liters.

$V_s$  = volume of "second" phase, in liters.

$HA_w$  = amount of free undissociated acid in aqueous phase, in gram molecules.

$HA_s$  = total amount of weak acid in the second phase, in gram molecules.

$BA$  = amount of salt of the weak acid, in gram molecules; which is equal to the amount of available base.

$A'$  = amount of dissociated acid anions in aqueous phase, in gram molecules.

$\gamma$  = degree of dissociation of the salt.

(Since by far the greater part of  $A'$  is derived from the dissociation of  $BA$ , Henderson showed that we can write  $A' = BA \gamma$ , without introducing any significant error. Furthermore,  $M = HA_s + HA_w + BA$  very nearly.)

$y$  = fraction of total acid existing in the aqueous phase =  $\frac{HA_w + BA}{M}$

$K$  = dissociation constant of the acid.

$K' = \frac{K}{\gamma}$

$[H]$  = concentration of hydrogen ions, in gram molecules per liter of aqueous solution.

(Concentrations for other constituents will also be expressed by brackets.)

$D$  = the distribution coefficient, applied here only to the molecular

species  $HA = \sqrt[n]{\frac{[HA_w]}{[HA_s]}}$

$C$  = a new factor to be defined below, related to  $D$ .

$C' = C \sqrt[n]{\frac{M}{M}}$ , a factor to be referred to as the distribution factor.

$n$  = the number of molecules of  $HA$  in an aggregate existing in the second phase, commonly written in the form  $(HA)_n$ .

We can now turn to a simple case. In addition to the conditions enumerated in the preceding paragraph:

For Case A it is further specified that the second phase consists of an inert solvent (gas or liquid), and that the weak acid,  $HA$ , in this phase is neither associated nor dissociated to any significant extent. The conditions at equilibrium can be defined by the following equations.

From the mass law:

$$[H] \times \frac{A'}{V_w} = \frac{K HA_w}{V_w}; \text{ or } [H] \times A' = K HA_w. \quad (1)$$

Since  $A' = BA \gamma$ , and  $K = K' \gamma$ , substituting we have:

$$[H] \times BA = K' HA_w \quad (\text{Henderson's equation})$$

or BA substitute its value  $yM - HA_w$  (definition of  $y$ ), and we have:

$$HA_w = \frac{y M [H]}{K' + [H]} \quad (2)$$

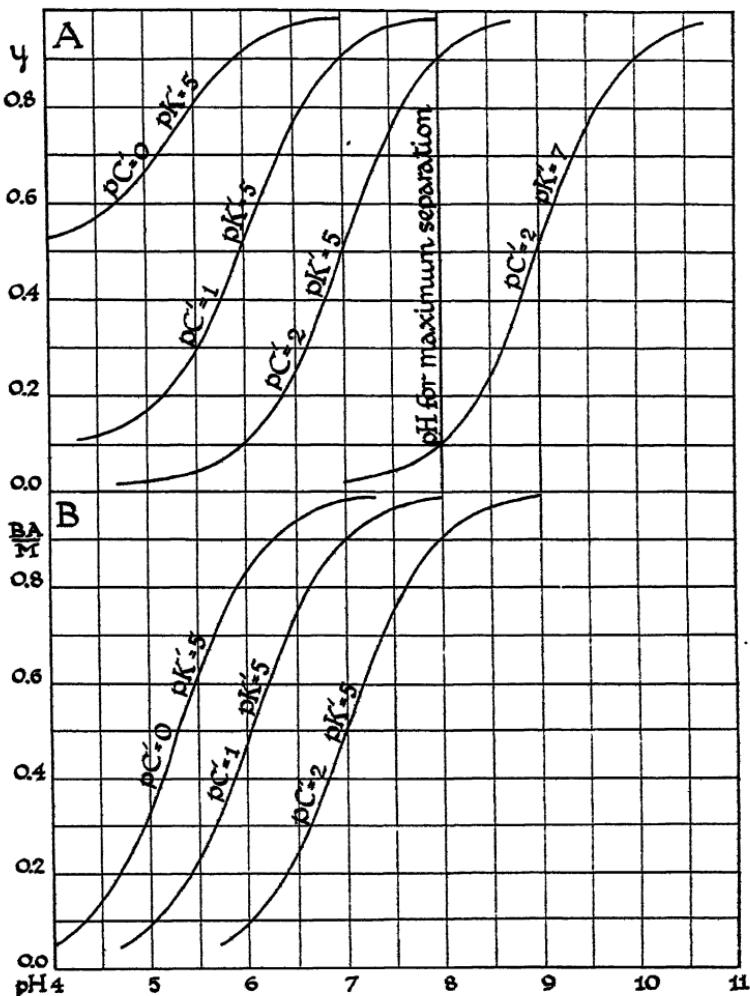


FIG. 1.

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*From the distribution law:*

$\frac{[\text{HA}_w]}{[\text{HA}_s]}$  = a constant,  $D$ , when the nature of the phases is specified. We will define  $C$  by the ratio  $\frac{\text{HA}_w}{\text{HA}_s}$ . That is:

$$C = \frac{\text{HA}_w}{\text{HA}_s} = \frac{[\text{HA}_w] V_w}{[\text{HA}_s] V_s} = \frac{V_w}{V_s} D \quad (3)$$

### *Case A.*

In Case A,  $n = 1$ , and  $C = C'$

From equation (3),  $\text{HA}_w = C \text{ HA}_s$ , and from the definition of  $y$ ,  $\text{HA}_s = M(1 - y)$ , therefore:

$$\text{HA}_w = C M (1 - y) \quad (4)$$

Combining equations (2) and (4),

$$\frac{y M [\text{H}]}{K' + [\text{H}]} = C M (1 - y), \text{ from which: } y = \frac{C (K' + [\text{H}])}{[\text{H}] + C (K' + [\text{H}])} \quad (5)$$

To find an expression for  $\frac{\text{BA}}{M}$ , substitute the term  $yM - \text{BA}$  for  $\text{HA}_w$ , in Henderson's equation, and we obtain:

$$\frac{\text{BA}}{M} = y \frac{K'}{K' + [\text{H}]} \quad (6)$$

and combining this equation with equation (5) to eliminate  $y$ , we get:

$$\frac{\text{BA}}{M} = \frac{CK'}{[\text{H}] + C (K' + [\text{H}])} \quad (7)$$

### *Case B.*

*Case B* is similar to *Case A*, except that the weak acid is associated to the extent  $n$  in the second phase, and exists in the form  $(\text{HA})_n$  almost entirely. Then, as Nernst has shown:

$$D = \frac{\text{HA}_w}{V_w} \times \frac{\sqrt[n]{V_s}}{\sqrt[n]{\text{HA}_s}} = \text{a constant. Since the volumes are constants,}$$

$$\frac{\text{HA}_w}{\sqrt[n]{\text{HA}_s}} = \text{a constant} = C, \text{ which applies only for the given volumes.}$$

(If  $D$  is known from previous experiments,  $C$ , for any given volumes can be found by multiplying  $D$  by  $\frac{V_w}{\sqrt[n]{V_s}}$ .)

It follows that (compare equation (4)):

$$\text{HA}_n = C \sqrt[n]{\text{HA}_s} = C \sqrt[n]{M(1-y)} \quad (8)$$

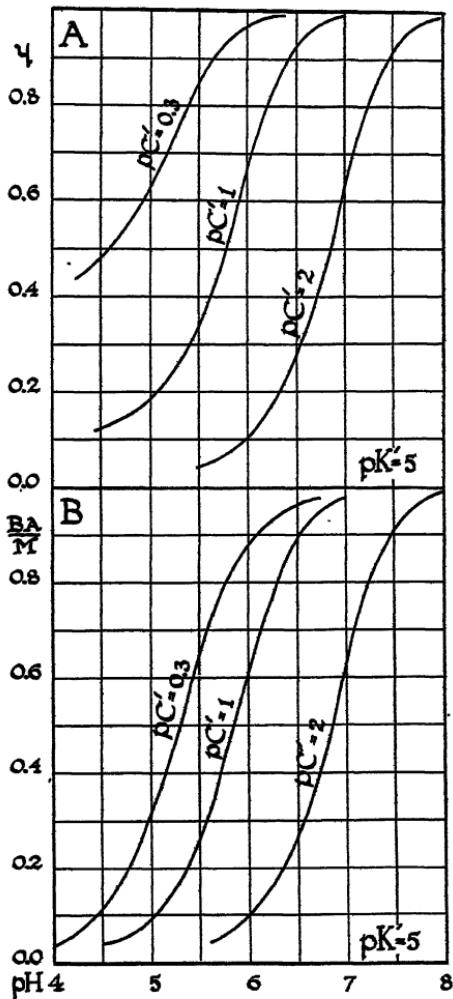


FIG. 2.

Combining equation (8) with equation (2) to eliminate the term  $\text{HA}_w$  we obtain:

$$\frac{y}{\sqrt[n]{1-y}} = \sqrt[n]{\frac{MC(K' + [\text{H}])}{M[\text{H}]}}; C' \text{ may be substituted for the term } C \frac{\sqrt[n]{M}}{M}$$

$$\text{This equation can also be written: } [\text{H}] = \frac{C' K' \sqrt[n]{1-y}}{y - C' \sqrt[n]{1-y}} \quad (9)$$

$$\text{Also: } \frac{\text{BA}}{M} = y \frac{K'}{K' + [\text{H}]} \quad (\text{compare with equation (6)}) \quad (10)$$

#### Case C.

For Case C it is specified that the second phase shall consist of pure acid only (i.e., as pure solid or liquid). An acid gas at constant partial pressure also satisfies the requirements.

In this case the acid can be considered as infinitely associated,  $n = \infty$ .

"D" =  $\frac{\text{HA}_w}{V_w} \times \frac{\sqrt[n]{V_s}}{\sqrt[n]{\text{HA}_s}} = \frac{\text{HA}_w}{V_w} = \text{the solubility of the acid in unit volume.}$

$$C = \frac{\text{HA}_w}{\sqrt[n]{\text{HA}_s}} = \text{HA}_w, \text{ so long as both phases are present.}$$

$$C' = C \frac{\sqrt[n]{M}}{M} = \frac{\text{HA}_w}{M}$$

Substituting  $C' M$  for  $\text{HA}_w$  in Henderson's equation, we obtain:

$$\frac{\text{BA}}{M} = \frac{C' K'}{[\text{H}]} \quad (11)$$

$$\text{Evidently, } yM = C + \text{BA}$$

$$\text{and, } y = C' + \frac{C' K'}{[\text{H}]} = \frac{C \cdot (K' + [\text{H}])}{[\text{H}]} \quad (12)$$

All the relations developed are for equilibrium conditions.

The peculiar characteristics of these cases are defined by an additional constant, designated  $C'$ . Since this constant will be referred to below, it will be convenient to mention, in words, the method of determining its value.

For any given system, the volume of the phases being kept constant,  $C$  measures the ratio of the amount of free weak acid in the aqueous phase to the  $n^{\text{th}}$  root of the total amount in the second phase. The value is, of

course, determined experimentally under conditions when no base is present. For a weak acid, the acid in the aqueous phase exists almost entirely in undissociated form,  $\text{HA}_w$ , when no base is present. In the second phase the acid is assumed to be almost entirely in the form  $(\text{HA})_n$ . Hence the justification for writing the value determined for  $C$  as equal to  $\frac{\text{HA}_w}{\sqrt[n]{\text{HA}}}$ , remembering that  $\text{HA}_w$  refers to the particular molecular species  $\text{HA}$ , whereas  $\text{HA}$  refers to the total acid in the second phase.

#### *Theoretical Discussion.*

Before discussing the special characteristics of these systems, we should consider a prediction advanced by Nernst (4), and quoted by Lewis (5). Nernst in his paper concluded that addition of HCl or of Na salicylate to a dilute aqueous solution of salicylic acid in equilibrium with a portion of benzene would *largely* increase the concentration of salicylic acid in the benzene phase (and, of course, in the aqueous phase). In the equations that have just been given, however, no account is taken of any such effect, since it is very small for weak acids and can be neglected when compared to the effects which have been or will be described. (Salicylic acid in normal concentration is about 3 per cent dissociated; increasing the hydrogen ion concentration to any possible extent would never result in more than about a 3 per cent increase in the concentration of the undissociated portion. The same reasoning applies to the effect of adding Na salicylate.) The *maximum* "Nernst effect" is 1 per cent (or less) if the ratio of the concentration of free weak acid to the value of its dissociation constant, *i.e.*  $\frac{[\text{HA}]}{K}$ , is  $10^4$  (or greater).

Accordingly, it can be said that the equations given represent the actual conditions underlying the important changes in the distribution of weak acids. It should be added that no account has been taken of changes which take place in respect to the nature of the phases. For instance, an aqueous solution saturated with  $(\text{NH}_4)_2\text{SO}_4$  cannot be substituted for a dilute aqueous solution if the constants, determined for the aqueous solution, are expected to hold. Similarly, salt formation in the aqueous phase, presumably affects the nature of this phase, and the distribution factors for acids may change considerably under the conditions which our equations are supposed to describe. In

any given case it should also be remembered that the salt formed may be soluble in the second phase.

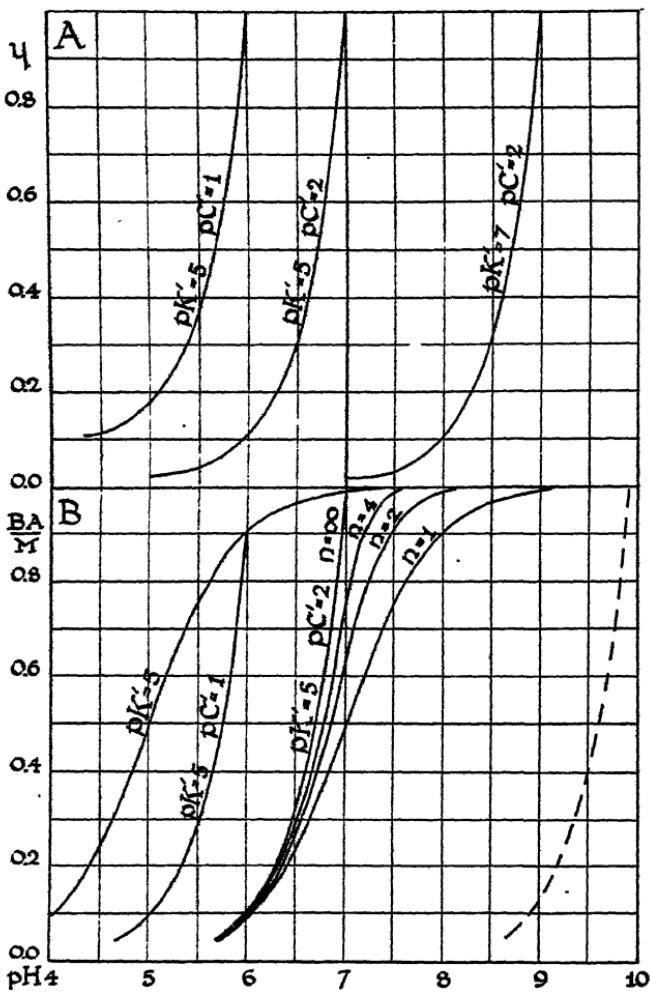


FIG. 3.

We can now examine any characteristics of the systems which appear to be of interest from the point of view of the acid-base equilibrium.

1. The effect of the second phase on the maximum molecular buffer value of a weak acid (Van Slyke's  $\beta_m$ (6)).

In Fig. 3 *B* a comparison can be made between the slopes of the four curves at the right of the figure. These curves represent acids whose dissociation constants are represented by the value  $pK' = 5$  ( $pX$  is short for  $\log \frac{1}{X}$ ), and whose distribution factors are represented by  $pC' = 2$ . The curve marked  $n = \infty$  illustrates the titration of a solution saturated with respect to the acid and containing a large part of undissolved acid besides. This system gives the highest buffer value. The curve  $n = 1$  gives the same slope as would be obtained for a curve representing the titration of any weak monovalent acid in simple aqueous solution, for example for the curve on the left of the figure. When  $n = 2$ , or  $n = 4$ , that is, for Case B, the slope is intermediate, between the slopes for Cases A and C. (The slope for Case B is difficult to define, since at any given point and with a constant value for  $n$  it depends in a somewhat complicated manner on the value of  $C'$  if  $C'$  is greater than about 0.1.)

2. The effect of the second phase on the pH at which the buffer value is a maximum. (It is suggested that this point be designated  $pH(\beta_{max})$ .)

It will be remembered, as was first shown by Henderson, that in simple aqueous solution a weak acid exhibits its maximum buffer action at a pH equal to  $pK'$ . This pH occurs at a stage in a titration when half of the weak acid has been "neutralized" by base; *i.e.*, when  $BA = HA$ .

When distribution takes place between two phases, however,  $pH(\beta_{max})$  is displaced by a certain definite amount. When  $C'$  is small: for all cases,  $pH(\beta_{max}) = p(C'K')$ . This value,  $pH(\beta_{max})$ , also measures the pH at which the change in the distribution of an acid is a maximum; or  $\frac{dy}{dpH}$  is a maximum when  $pH = p(C'K')$ . If  $C'$  is not small, then we have the following relations:

$$\text{For Case A: } pH(\beta_{max}) = p \left( \frac{C' K'}{1 + C'} \right), \text{ and here } \frac{BA}{M} = 0.5$$

For Case C:  $\text{pH}(\beta_{\max}) = p \left( \frac{C' K'}{1 - C'} \right)$ , and here  $\frac{BA}{M} = \frac{C'}{1 - C'}$

For Case B:  $\text{pH}(\beta_{\max})$  has a value between the two values given above.

When  $C'$  is small, at the point  $\text{pH}(B_{\max})$ ,  $\frac{BA}{M} = 0.62$

when  $n = 2$ , and 0.72 when  $n = 4$ .

### 3. The effect of the second phase on the apparent strength of a weak acid.

No general rule can be laid down for the three cases. For any case it is evident that, if distribution takes place, the concentration of the acid is effectively diminished, as far as the aqueous phase is concerned. For Case A we can make the more precise statement: Those properties of a weak acid in simple aqueous solution which are characterized by the dissociation constant  $K$ , are characterized by  $\frac{C'K}{1+C'}$  (or  $C'K$  if  $C'$  is small) when the same acid is subject to distribution effects described under Case A and defined by the constant  $C'$ . This statement involves the relations brought out in the two preceding sections.

If  $C'$  is small, all the effects described can be expressed quantitatively, for all the cases discussed, and for the given point  $\text{pH}(\beta_{\max})$ , by substituting the value  $pC'K'$  (when distribution takes place) for the value  $pK'$  (applying to simple aqueous solution). In other words, if an acid is distributed between two phases, it behaves as a much weaker acid, its buffer value is sometimes changed, and the pH at which the buffer value is a maximum is shifted toward the alkaline side. (These statements, and certain relations developed, do not apply to conditions where  $C'$  is greater than 1.)

#### EXPERIMENTAL.

To test the validity of the foregoing equations simple systems, typifying the three cases discussed, were selected. It happens that other systems which would exhibit more strikingly the peculiar characteristics of the two-phase equilibrium are more apt to involve, perhaps by their very nature, complicating factors and analytical or other experimental difficulties.

As an example of Case A, the distribution of lactic acid between amyl alcohol and aqueous solutions was studied. Benzoic acid distributed between benzene and aqueous solutions afforded an example of Case B, and crystalline benzoic acid in equilibrium with an aqueous solution corresponds to Case C.

*Case A.*

The distribution ratio of lactic acid (Merck's) between equal volumes of amyl alcohol and water was determined at three concentrations (1, 0.2, and 0.02 N volume concentrations with respect to the volume of one phase) and found to be a simple constant,  $\frac{HA_w}{HA_s} = 1.9$ . In further experiments the volume of amyl alcohol was four times greater than the volume of water, hence the value for the distribution factor,  $C = C' = 0.475$ .

TABLE I.

$(1 - y)$	$y$	$\frac{BA}{M}$	pH
0.678	0.322	0.0	
0.535	0.465	0.2	3.39
0.333	0.667	0.5	3.97
0.180	0.820	0.75	4.46
0.106	0.894	0.9	4.78

80 cc. portions of amyl alcohol, containing lactic acid (0.05 N volume concentration), were shaken up in separate cylinders with 20 cc. portions of aqueous solutions of NaOH of varying concentrations. After equilibrium had been attained, the acid remaining in the alcohol layer was estimated by titration of a 10 cc. portion (diluted with ethyl alcohol) with aqueous NaOH, using phenolphthalein as an indicator. The pH of the aqueous layer was then determined electrometrically. The results are given in Table I.  $(1 - y)$  is the fraction of the total acid in the alcohol layer at equilibrium,  $y$  is the total lactic acid or lactate in the aqueous portion.  $\frac{BA}{M}$  is the amount of NaOH originally present in the aqueous portion expressed as the fraction of the amount required to neutralize all the acid present.

To compare these results with calculated values it is necessary to know the value of  $pK'$  for lactic acid. This was determined, for our sample of the acid, by measuring the pH of a 0.2 N solution of the acid to which had been added half its volume of a 0.2 N solution of NaOH. The observed value was  $\text{pH} = 3.54 = pK'$ .

Using the values for  $C'$  and  $pK'$  which have been given, the curves in Figs. 4 A and 4 B have been calculated. The distribution curve, Fig. 4 A, is calculated from equation (5). In Fig. 4 B, Curve 1 is the theoretical titration curve for lactic acid in aqueous solution, and Curve 2 is the curve which applies to the experiment, calculated by means of equation (7). The x's, plotted from the observed data for the distribution curve, are taken from values for  $y$  and pH in the table, and for the titration curve they are taken from the values for  $\frac{BA}{M}$  and pH.

### Case B.

It is well known that the distribution relations of benzoic acid between benzene and water can be calculated on the basis that benzoic acid in benzene exists in the form  $(\text{HA})_2$ . Hence  $\frac{\text{HA}_w}{\sqrt{\text{HA}_s}}$  is a constant ratio =  $C$  (which applies only to given volumes of the two phases). To determine the value of  $C$ , 20 cc. of benzene, containing 0.002 gm. molecules of benzoic acid, were shaken up with 20 cc. of water. At equilibrium 0.000152 gm.-molecules of the acid were in the water phase, and 0.00185 in the benzene. Hence

$$C' = C \frac{\sqrt{M}}{M} = \frac{0.000152}{\sqrt{0.00185}} \times \frac{\sqrt{0.0002}}{0.0002} = 0.079$$

The same volumes of benzene and water, and the same amount of benzoic acid were used in the series of experiments which are summarized in Table II. In the experiments marked with an asterisk (\*) NaOH was present in the aqueous portion; in those marked with a dagger (†) buffer salts were also present. The latter do not yield data for the titration curve, but show that the distribution curve can be expressed as a function of the pH and is independent of the nature of the salts which may in part determine the pH.

$pK'$  for benzoic acid was determined as for lactic acid except that the concentration had to be sufficiently low (0.02 N) to insure complete solution of the acid. The value for  $pK'$  was found to be 4.05.

Figs. 4 C and 4 D show the theoretical curves and the observed points. Curve 1, Fig. 4 D, is the theoretical curve for the titration of benzoic acid in aqueous solution, Curve 3 the theoretical curve for the series of experiments of Table II. These curves are calculated from equations (9) and (10).

TABLE II.

$(1 - y)$	$y$	$\frac{BA}{M}$	pH
0.917*	0.0825	0.0	3.40
0.880*	0.120	0.05	4.04
0.780*	0.220	0.15	4.37
0.616†	0.384		4.78
0.575*	0.425	0.40	4.88
0.334†	0.666		5.00
0.255*	0.745	0.70	5.22
0.150†	0.850		5.40
0.048*	0.952	0.95	5.77

TABLE III.  
Total acid ( $M$ ) = 0.005, solubility = 0.00119.

	$\frac{BA}{M}$	pH
Crystals present.....	0.1	3.68
" " .....	0.2	4.04
" " .....	0.5	4.45
All dissolved.....	0.8	4.62

## Case C.

Portions of benzoic acid (0.005 gm. molecules each) were introduced into 50 cc. volumetric flasks, which had been filled up to the mark with aqueous solutions containing various amounts of NaOH. After equilibrium had been attained (*i.e.*, after gentle warming and cooling to 20°) the pH of samples from each flask was determined colorimetrically (checked by several individuals), using Clark's series of standards, freshly made up according to

the procedure given in Clark's book. The solubility ( $C$ ) of benzoic acid given in the tables is 0.00119 gm. molecules in 50 cc.

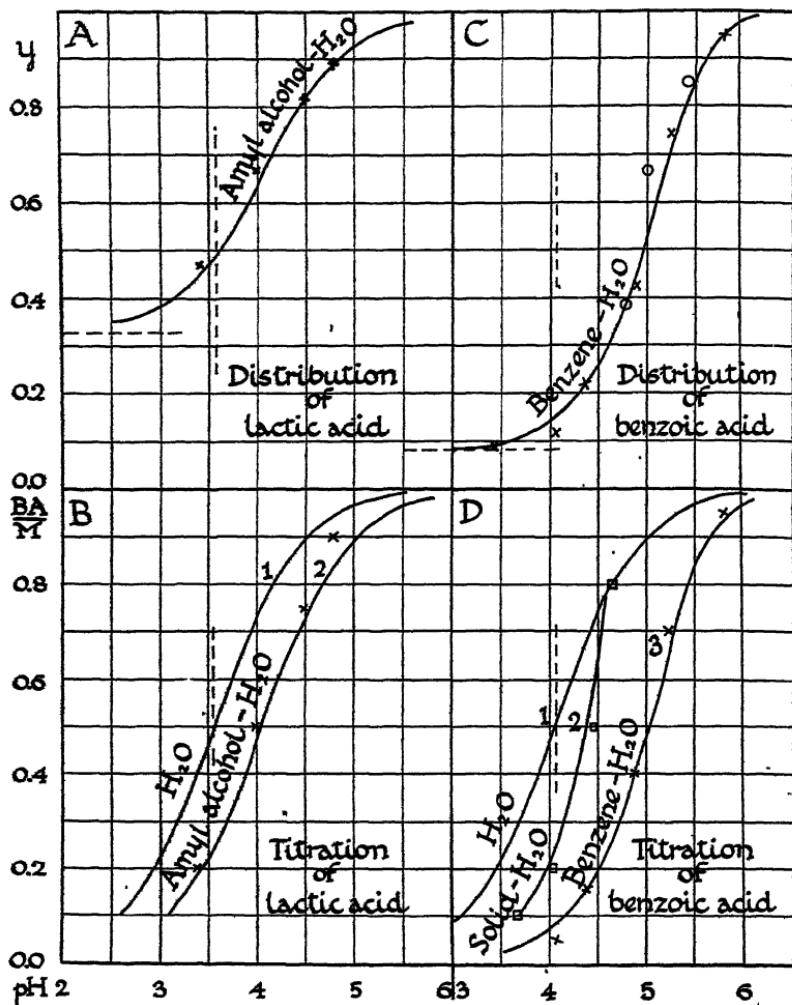


FIG. 4.

at 20°, hence  $C' = \frac{0.00119}{0.00500} = 0.238$ , and, as before,  $pK' = 4.05$ . Table III gives the results observed.

Curve 2, Fig. 4 D, is the calculated curve, from equation (11), representing the relations which obtain when both phases are present. At the point  $\text{pH} = p\left(\frac{C'K'}{1-C'}\right)$ , that is  $\text{pH} = 4.55$  in this example, the second phase disappears, and the relations which hold for the titration in simple aqueous solution (one phase) are sufficient. The observed points are marked by squares in the figure. It is interesting to note that the point  $\text{pH} = p\left(\frac{C'K'}{1-C'}\right)$  also represents the pH at which the maximum buffer value of the acid is exhibited (although, actually at this point, the high buffer value is only effective in one direction). This is also, obviously, the pH at which the acid begins to precipitate when a strong acid is added to a solution of the salt.

It appears, from these experiments, that no fundamental error is involved in the equations which have been submitted, nor would this have been expected, since the equations are based on sound generalizations which are easily combined for the purpose in hand. No claim is made that these equations are now in their most useful form, but they serve, at any rate, to indicate the general effects to be encountered when dealing with simple two-phase systems.

#### *Further Considerations.*

It remains for us to suggest the significance, if any, of the foregoing results, and to point out a few applications which may be of use.

A glance at the curves makes it apparent that we can separate two acids if their dissociation constants are sufficiently different, even though their distribution coefficients and solubilities in various solvents are identical. The separation becomes more and more complete as the values  $pC'K'$  for the acids differ more and more from each other. The values of  $C'$  should be small, but this can usually be provided for by increasing the volume of the second phase. Fig. 1 A, considering the two curves at the right, will illustrate the method of separation, which is based on the fact that the pH of the aqueous phase can be controlled by means of a suitable indicator and that a large shift of acid from one phase into another occurs within a relatively narrow range of

variation of pH. The two acids, the behaviors of which are represented by the curves, have values of  $pC'K'$  equal to 7 and 9. The pH at which maximum separation is obtained is therefore 8. Suppose that the acids are dissolved in water, and that they are to be separated by means of benzene as the second solvent.

Equilibrium is first attained between the two phases, and alkali added until the pH of the aqueous phase reaches 8. The two phases are shaken up together again, acid passes into the aqueous portion, and more alkali is needed to bring the pH back to 8. When equilibrium is obtained at a pH of 8, the two phases are separated. At this stage we should have 90 per cent of the strong acid in the aqueous phase and 10 per cent of the weaker acid. If the aqueous phase is now extracted with a fresh portion of benzene, and the pH at equilibrium brought to 8 (this time by adding HCl), then 81 per cent of the strong acid will remain in the aqueous phase and 1 per cent of the weak acid. One more extraction will yield 72 per cent of the original amount of the strong acid and 0.1 per cent of the weak acid. The weak acid may be similarly purified by extracting the first separated portion of benzene with fresh portions of water, always bringing the system to equilibrium at a pH of 8 to obtain the most efficient separation.

The same method can be used to separate two acids by precipitation from solutions of their salts. This is illustrated by the two curves at the right of Fig. 3 A. As strong acid, HCl, is added to such a solution, the weak acid whose  $pC'K'$  value is 9 will begin to precipitate when the pH of the solution drops to 9, and will continue to precipitate pure until the pH has dropped to 7. At this stage the stronger acid would begin to precipitate, and the time for separation is reached. 98 per cent of the weaker acid can be filtered off, presumably quite pure; but further efforts to purify the stronger acid will be useless, because the curve for the weaker acid which remains now follows the curve for the stronger acid. What is gained in favor of the purification of the weaker acid is lost in respect to the stronger acid, as far as this precipitation method is concerned.

Similar principles would apply to the fractional precipitation of insoluble metallic salts of weak organic acids, and it seems that a judicious use of indicators in such procedures might serve to make certain separations more precise.

Since the distinctive features of the systems we have been discussing are most evident when small values of  $C'$  are encountered we would expect that the principles discussed would be profitably turned to account in the study of systems involving the higher fatty acids. As these acids are extremely insoluble in water, we would *expect* to obtain a titration curve such as the dotted curve in Fig. 3 B, if we assume that the soap formed is soluble in water. Under these conditions the insoluble fatty acid would presumably be emulsified in the soap solution, and would give to these solutions their colloidal properties. The possibility that the solubility of the fatty acid would be significantly increased in this condition should not be overlooked. The value for  $pC'K'$  for which the tentative curve is drawn is taken from McBain's data (7) for the pH of a "solution" 1 N in respect to Na palmitate and palmitic acid each. (This gives a value of 9.9 for  $pC'K'$ .)

Studying the distribution of oleic acid and oleate between benzene and water at various equilibrium hydrogen ion concentrations, I have found that when the total oleic acid is equally distributed between the two phases, the pH of the aqueous phase is 9.6. If the pH is less than 9.0, the oleate in the aqueous phase is practically zero; *i.e.*, there is not a sufficient concentration to lower the surface tension of the water phase. Hartridge and Peters (8) have studied the surface tension at the interface of an aqueous solution at various pH's and purified olive oil. They found the surface tension at the interface to be a linear function of the pH over a considerable range, dropping to zero as the pH reached the value 9.

McBain alludes to the fact that  $\text{CO}_2$  will decompose soap solutions, so that ultimately nearly all the fatty acid may be removed. He adds that the equilibria involved have not received quantitative study. Since the  $pK'$  values for higher fatty acids are thought to be of the order 5, the action of  $\text{CO}_2$  might appear at first sight to be somewhat anomalous; but from what has been said it will be noticed that the effective strength of these acids is measured by the value  $pC'K'$  (roughly), a value of the order of 10 for 0.1 N solutions. In short the higher fatty acids, in aqueous solution at least, behave like acids very much weaker than  $\text{H}-\text{HCO}_3$ .

The possibility suggests itself that the solubility of the soaps is limited. If this is true, then, as long as undissolved soap and undissolved acid are present in equilibrium with a given solution, we have  $\frac{HA}{BA} = \text{a constant}$ . Addition of a portion of HCl to the system results in a transfer of fatty acid from the undissolved soap to the undissolved fatty acid and leaves the solution unchanged (at equilibrium) except for the formation of a certain amount of NaCl. Hence there is a unique hydrogen ion concentration at which the three phases can exist in equilibrium, and we have a system which might truly be termed a perfect buffer system.

Another application of the relations involved in the distribution of acids suggests itself from an analogy which can be drawn between the systems we have reviewed and typical systems which have been used to investigate the Donnan equilibrium. One essential feature of this equilibrium is that it is a two-phase equilibrium, and another is that one reacting ion is present in one phase only (more precisely, one ionic species cannot pass from one phase to the other). These two features are closely paralleled by a system consisting of two aqueous solutions separated

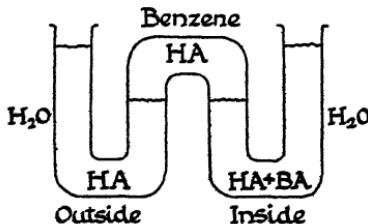


FIG. 5.

by a layer of some immiscible solvent such as benzene, the one solution containing an acid (soluble in benzene) and the other containing the same acid in equivalent concentration plus some of the salt of the acid. The benzene layer will contain only the free acid, and the system will be in equilibrium with respect to the acid. A neutral substance, such as sugar, can be added to the solution which contains only the free acid, and the system will then be in osmotic equilibrium.

In such a system the Na ion corresponds to the indiffusible ion, and, to compare this system with those that Loeb has investigated, we can call the solution containing the salt of the acid the inside solution (Loeb had a solution containing positive gelatin ions) and we can call the other the outside solution.

At equilibrium the following relations will hold:

$$\begin{array}{ccc} \text{Outside.} & \text{Membrane.} & \text{Inside.} \\ H \times A = K \cdot HA_w & = CK \cdot HA_s & = HA_w \cdot K = H \times A \end{array}$$

which might be represented numerically by the figures:

$$10 \times 10 = 100 = X = 100 = 1 \times 100 (+ \text{Na} = 99)$$

Evidently the inside solution possesses the greater osmotic pressure, a greater concentration of acid anions, and a lower hydrogen ion concentration. Further work will be attempted with this system as it is thought that the system described offers a relatively simple case of the Donnan equilibrium, and offers an opportunity for measuring such properties as the interfacial surface tensions, and the cataphoretic charges of benzene droplets in either of the two aqueous solutions with which these droplets are in equilibrium.

In physiology, it seems that a use could be found for the equations that have been developed here. The conditions governing the physicochemical behavior of fats, fatty acids, and soaps in the intestine are in need of more precise definition. Very roughly we might hazard the prediction that soap from higher fatty acids begins to be formed in the intestine when the pH has reached the value 9. For a more exact evaluation we must accumulate more data, for instance data to define the conditions under which calcium soaps, slightly soluble in fat, are formed.

This brings up the problem of the Na:Ca ratio in the blood. Are calcium soaps present dissolved in blood fat, and is the distribution of calcium between blood fat and plasma (and coincidentally the Ca ion concentration in the plasma) affected by physiological changes of pH, should such a distribution really exist? Calcium salts in general, so many of which are insoluble, offer interesting material for physiological investigation from the point of view of the acid-base equilibrium, especially since the

physical state of calcium compounds must have a bearing on calcium assimilation from the intestine. In this connection reference should be made to an article by Wendt and Clarke (9) which has just been published, and which gives the titration curve for the neutralization of  $H_3PO_4$  by  $Ca(OH)_2$ . At first sight the result is somewhat unexpected, and can be summed up approximately by saying that as  $Ca(OH)_2$  is added to the solution of  $H_3PO_4$  there is a sudden change of pH when one-third of an equivalent of base has been added. Soon after, a precipitate of tricalcium phosphate (perhaps of dicalcium phosphate also) appears and the pH remains practically unchanged at a value about 5.8 until 0.8 equivalents of base have been added, (no sudden change occurring when two-thirds of an equivalent are present). At this point the solution becomes gradually more alkaline, and finally suddenly much more alkaline at the end-point. Saturated lime water and 0.05 N  $H_3PO_4$  were the solutions used for the titration. Equilibrium in this system, involving three phases or at least two, was only reached after considerable time. Presumably the value 5.8, for the pH at which the buffer action was a maximum, would shift toward the alkaline side if lower concentrations were used. While the pH remained constant, it is possible that addition of  $Ca(OH)_2$  was causing little or no change in the *solution*, but merely a transfer of precipitated dicalcium phosphate to precipitated tricalcium phosphate. The solution, saturated with respect to the two salts, is a perfect buffer with a pH 5.8 as long as the three phases are present.

#### SUMMARY.

The distribution law and the law of mass action (Henderson's equation) have been combined to yield equations expressing the relation between the distribution of a weak acid between two phases, one of which is an aqueous solution, and the pH of the aqueous phase.

By the use of an easily determined constant, designated  $C'$ , we can in general express by the value  $C'K$  certain properties of a weak acid subject to distribution between two phases; namely, those properties which are defined by the value of  $K$ , the dissociation constant, when the acid exists in simple aqueous solution.

Some consequences deduced from the equations are discussed in some detail in respect to their bearing on the acid-base equilibrium in general. Thus the buffer value and the change in the actual distribution of total acid between the two phases are a maximum when the pH of the aqueous phase is equal to  $pC'K'$  (when  $C'$  is small). The value  $pC'K'$  measures the apparent strength of the acid under the conditions specified, and it defines the pH at which a relatively insoluble weak acid begins to precipitate when a strong acid is added to a solution of the salt of the weak acid.

Experimental data are given to support the main conclusions.

Suggestions are made concerning the application of the equations to problems involving the separation of weak organic acids from one another. Speculations are advanced concerning possible relationships between soap solutions and systems described in this paper. A system involving the Donnan equilibrium and the equilibria discussed in the paper (the two equilibria involved are closely related) is described.

Some problems in physiology which might reasonably be approached from the point of view expressed here are suggested.

I am deeply indebted to Dr. E. L. Scott, under whose kindly vigilance this work was undertaken, to Prof. J. M. Nelson for frequent criticism and advice, and to Prof. H. B. Williams for the privilege of working in his department and for the encouragement he has afforded me throughout.

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## OBSERVATIONS ON THE REGULATION OF OSMOTIC PRESSURE (CONDUCTIVITY, CHLORIDES, FREEZING POINT, AND PROTEINS OF SERUM).\*

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In previous reports (Gram and Norgaard, 1) it has been shown that there is a remarkable constancy in the ratio between the NaCl of the serum and the conductivity. In several cases in which there was a marked decrease in conductivity there was a proportionate drop in the NaCl concentration. This observation has led to further investigation concerning the frequency and occurrence of decrease in the conductivity of serum, and also of the constancy of the chloride ratio in cases with decreased conductivity.

In establishing the accuracy of the ionometer method by a comparison with the standard conductivity method (Gram and Cullen)<sup>1</sup> it was found that the depression of the conductivity due to the proteins was 2.2 per cent of the total value per 1 per cent protein, with both methods, which agrees fairly well with the figure given by earlier authors (Bugarszky and Tangl, 2).

In some of the diseases which are included in this survey there are known to be marked fluctuations in the protein content, wherefore it seemed desirable to correct the conductivity data for the protein influence. When this was done the constancy of the new ratio  $\frac{\text{NaCl titrated}}{\text{Corrected NaCl equivalent of conductivity}}$  was somewhat more marked than the constancy of the uncorrected ratio.

\* Aided by a grant from the Robert M. Girvin Foundation.

<sup>1</sup> Gram, H. C., and Cullen, G. E., *J. Biol. Chem.*, 1923, in press.

When it had been established that marked changes did occur the reason for these conductivity changes in various widely different conditions was sought. Cryoscopic determinations of the freezing point depression seemed to be the key to the question inasmuch as the low conductivities were divided into two groups, the first (pneumonia and some nephritis cases) showing a decreased freezing point depression while the second (high sugar diabetes and uremias) showed an increase in this value. The characteristic of the latter group was an increase of the non-electrolyte solutes of the serum.

#### *Technique.*

*Taking of Blood.*—The blood drawn was from a cubital vein by suction into a 50 cc. centrifuge bottle. In dripping through the air to the bottom of the bottle some of the CO<sub>2</sub> presumably was lost. In order to test the influence of this loss of CO<sub>2</sub> on conductivity a series of bloods was taken under oil and half of each specimen was immediately expelled through the needle and allowed to drip through the air into another bottle. Both portions were centrifuged and the conductivity determined with the results shown in Table I. In only one case did there occur a discrepancy between the two portions beyond what might be expected from the mean error of the method (see below). The electrolyte shift between cells and serum due to loss of CO<sub>2</sub> is, therefore, within the limit of error of the ionometer method.

*Conductivity.*—The ionometer of Christiansen (3) was used. In a few early determinations an apparatus for 220 volts d. c. was used, while all the rest were done with another instrument at 110 volts d. c. Several readings were taken on each serum, always at a temperature of 20°C. Conductivity was expressed in terms of sodium chloride equivalents according to a calibration curve constructed by the use of pure NaCl solutions. A comparison of the accuracy of the instrument as compared with the standard method is reported elsewhere. The mean error by the first instrument was found to be  $\pm 0.0033$  gm. of NaCl (equivalent), by the second instrument  $\pm 0.0028$  gm. of NaCl (equivalent).<sup>2</sup>

<sup>2</sup> Ten determinations on ten specimens. Calculation from equation:

$$\mu = \sqrt{\frac{\sum d^2}{n-1}}$$

*Chlorides.*—The Austin and Paul modification<sup>3</sup> of McLean and Van Slyke's method (4) was used. The proteins were precipitated by picric acid. The solutions were standardized by using the same NaCl solutions used for standardizing the ionometer. In comparing the results in this paper with those of previous papers (Gram and Norgaard, 1) it must be remembered that the chlorides in the earlier papers were determined by the Bang (5) method (grams of NaCl per 100 gm.) and the percentage values would, therefore, be slightly higher. Also, in the earlier papers

TABLE I.  
*Conductivity of Serum from Duplicate Specimens Respectively Taken under Oil and Exposed to Air.*

Name.	Sex.	Diagnosis.	NaCl equivalent of conductivity.	
			Air.	Oil.
We.	M.	Bronchial asthma.	0.659	0.659
Ai.	F.	Diabetes.	0.609	0.613
Le.	M.	Gastric neurosis.	0.644	0.632
			0.644	0.640
Ha.	"	Pernicious anemia.	0.659	0.659
Do.	F.	Nephritis.	0.628	0.632
Pu.	M.	Convalescent after pneumonia.	0.647	0.647
Pa.	"	Epilepsia.	0.659	0.659
La.	"	Nephritis.	0.678	0.678
<i>Average</i> .....			0.644	0.643

hirudin plasma was used and the conductivity of serum must be expected to be slightly higher than that of hirudin plasma, because of the absence of fibrinogen in the serum. The mean error on the chloride determination was found to be  $\pm 0.006$ .

*Proteins.*—The determination was done by the refractometer of Abbé with a running water bath at 17.5°C. The refractometer was corrected by determining the refraction of freshly distilled water which should be 1.33320. The corrected refractive index was converted into terms of protein percentage by a curve drawn from the figures given in Domarus' handbook (6). The mean error was found to be  $\pm 0.10$  per cent protein. The correction of

<sup>3</sup> Austin, J. H., and Paul, J. R., unpublished data.

conductivity for protein depression was that determined by Gram and Cullen:<sup>1</sup>

$$\text{Corrected conductivity} = \left\{ \frac{\text{Observed conductivity}}{100 - (2.2 \times \text{protein percentage})} \right\} \times \frac{100}{100 - (2.2 \times \text{protein percentage})}$$

*Freezing Point.*—The cryoscope of Burian and Drucker (7) was used. The average of two determinations is given, the zero point being taken as the freezing point of pure distilled water determined daily by the same method. A motor connected with pulleys was used for stirring the liquid and the bath. With hand stirring the mean error was found from ten determinations to be twice as large ( $\pm 0.0084^{\circ}\text{C}.$ ) as that found when using mechanical stirring ( $\pm 0.0042^{\circ}\text{C}.$ ). Each freezing point depression is the average of two determinations.

### Results.

*Conductivity.*—Fig. 1 shows the uncorrected conductivities of all the cases examined; they are also given in the tables. It will be seen that the conductivities of thirty normal sera varied between 0.674 and 0.640 NaCl equivalent,<sup>4</sup> with an average of 0.658. 76 miscellaneous cases—including diseases of the nervous system, heart, muscles, joints, ductless glands, and various medical diseases not included in the other groups—show only one variation beyond the normal limits, this being a decrease in a case of colitis.

A case of diabetes insipidus which, however, was under treatment with pituitrin showed no changes. Among the nephritis which included some cardiorenal cases, sixteen showed normal values, while five were above and three below the normal.

Excluding pneumonia we found in various infections seven out of nineteen with conductivities below the normal. In pneumonia twelve out of fifteen showed decreased conductivities, and in most of them the decrease was marked. Grouping together the cases of diabetes that at our first examination showed blood sugar less than 0.150 per cent, no glycosuria, and no ketonuria, we find that ten out of eleven had normal conductivities while one showed an increase. In diabetes with high blood sugar, with or

<sup>4</sup> Translation into terms of specific conductivity at 20°C. can be effected by a curve published by Gram and Cullen.<sup>1</sup>

without acidosis, seventeen cases had low conductivities, one high, and three normal. Some of the cases of diabetes have been studied several times during dietary and insulin treatment and these examinations have been put in a class by themselves.<sup>5</sup>

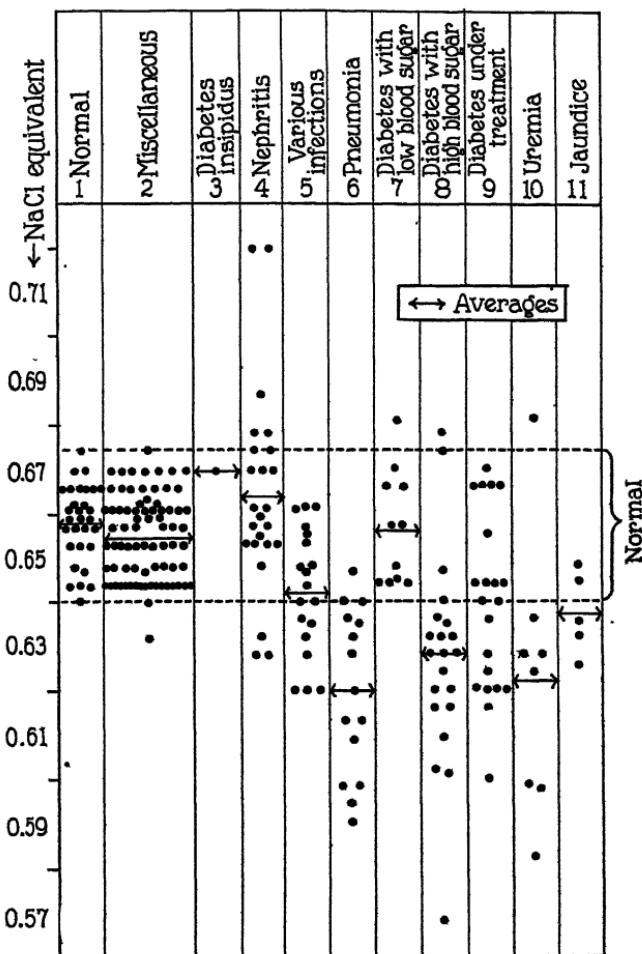


FIG. 1. NaCl equivalent of conductivity in various sera.

<sup>5</sup> The reasons for placing the repeated examinations in diabetes in a special group are the following: (1) Very large fluctuations in the protein content of serum occur especially during insulin treatment. (2) It is

Twelve examinations showed normal values, while the remaining nine were low. Of uremics, one case showed a high value, while the remaining seven were low. Out of five examinations in jaundice, three were low and two were normal.

*NaCl: Conductivity Ratio.*

A previous investigation with Norgaard brought out the fact that there is in the case of normals, nephritis, and pulmonary infections a striking agreement between the changes in the conductivity and in the titrated chlorides of hirudin plasma. It remained to be seen whether the same relation holds in the present series, in which other types of disease showing altered conductivities are included. The results of the chloride titrations (in grams of NaCl per 100 cc. of serum) are given in the tables and also in Fig. 2. The relation between titrated chloride and conductivity is shown in Fig. 3. The diagonal line in this graph represents the average normal ratio,  $\frac{\text{NaCl percentage}}{\text{NaCl equivalent}} = 0.917$ , and the outlined rectangle represents the range of variations in normal individuals. The tendency of the points plotted to fall on the diagonal line is clearly indicated though the pathological values show a greater deviation than the normal. The individual ratios will be found in the tables.

In the above mentioned paper with Cullen the depression of the conductivity due to protein was found to be 2.2 per cent of the total value per 1 per cent protein. Fig. 4 shows the protein percentages found in the different groups, showing that many of the pathological cases had a low protein concentration, while the high protein concentration was rare. (See also the tables.)

If we introduce the correction given above we approximate the NaCl equivalent of the conductivity of the salts of the serum, as it would be if no proteins were present. These corrected values are found in Fig. 5 and also in the tables. It will be seen that the general trend in the various groups is the same. As a matter of

possible that the regulation of salts is not prompt enough to follow the sudden change in blood sugar produced by insulin treatment. (3) It is desirable to avoid obscuring the picture by the frequent repeated examinations in cases showing unusual changes.

fact some high conductivities in diabetes in which there was a diminished protein concentration are brought down by the correction. The results clearly prove—as would be expected from the chloride determinations—that the low conductivities are not

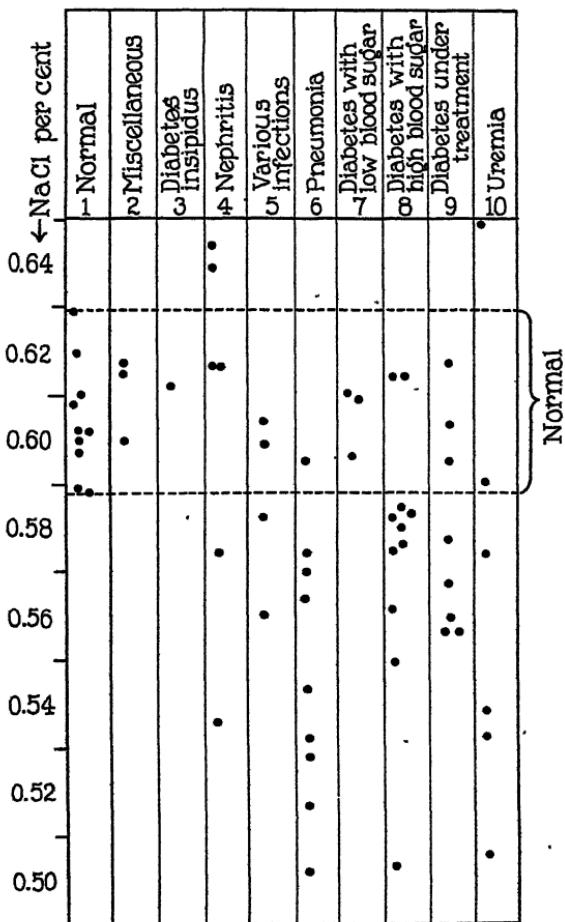


FIG. 2. Chlorides (titrated) in various sera.

due to an increase of proteins. The corrected conductivity equivalents in normals vary between 0.811 and 0.787, with an average NaCl equivalent of 0.799. If these corrected conductivity values are compared with the titrated chloride values we get

the results shown in Fig. 6 where the diagonal line represents the average normal ratio  $\frac{\text{NaCl percentage}}{\text{Corrected NaCl equivalent}} = 0.755$  and the outlined area encloses the range of normal variations. (See also the tables.) It will be seen that the points plotted will tend

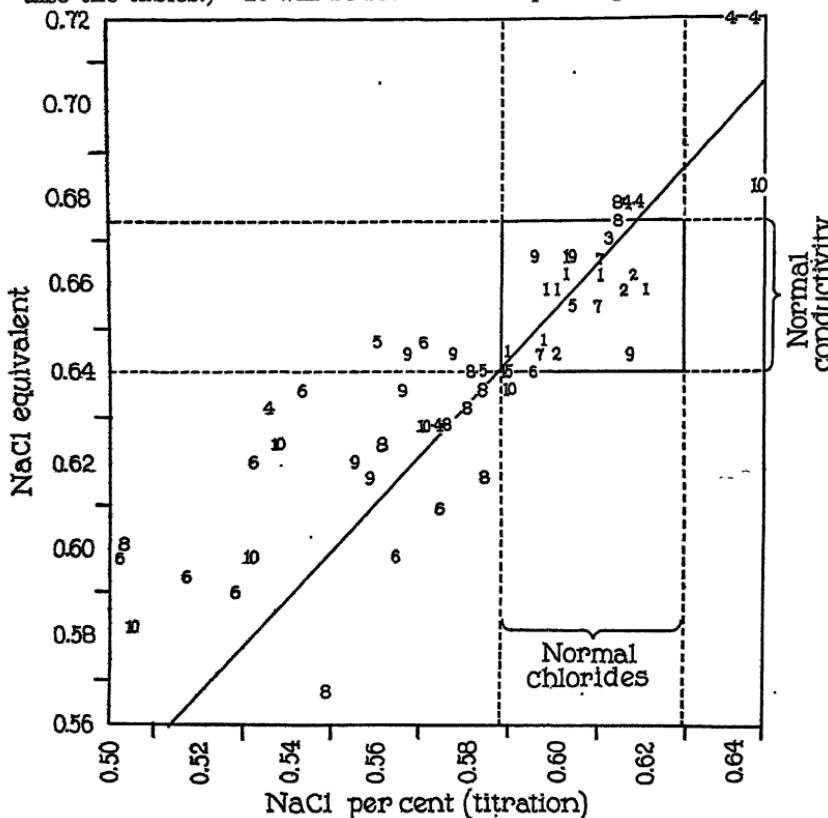


FIG. 3. Relation between NaCl equivalent of conductivity and titrated NaCl in various sera. The dotted lines represent the normal boundaries. The diagonal line represents the average normal ratio

$$\frac{\text{NaCl titrated}}{\text{NaCl equivalent}} = 0.917$$

In this and the following figures, the figure 1 indicates normal cases, 2 miscellaneous, 3 diabetes insipidus, 4 nephritis, 5 various infections, 6 pneumonia, 7 diabetes with low blood sugar, 8 diabetes with high blood sugar, 9 diabetes under treatment, 10 uremia, 11 jaundice.

*Normal Individuals.*

No.	Name.	Sex.	Date.	NaCl equivalent of conductivity.	NaCl percentage (titrated)*.	Ratio: NaCl percentage NaCl equivalent.	Δ - °C. (cryoscope).	Protein percentage (refractometer).	NaCl equivalent corrected for protein influence.	NaCl percentage	Ratio: Corrected NaCl equivalent
			1928								
1	T. U.	F.		0.670							
2	A. N.	M.		0.670							
3	E. H.	F.		0.666							
4	S. M.	M.		0.666							
5	H. H.	"		0.666							
6	A. N.	"		0.666							
7	E. C.	F.		0.666							
8	J. A.	M.		0.661							
9	L. H.	"		0.661							
10	A. F.	F.		0.661							
11	A. J.	M.		0.657							
12	A. S.	F.		0.657							
13	P. H.	M.		0.657							
14	T. O.	F.		0.657							
15	V. J.	M.		0.653							
16	K. B.	F.		0.653							
17	V. P.	"		0.653							
18	T. O.	"		0.648							
19	E. R.	M.		0.644							
20	D. L.	F.		0.644							
21	J. A.	M.	Feb. 3	0.674	0.629	0.93	0.565	7.7	0.811	0.77	
22	H. C.	"	" 7	0.659	0.600	0.91	0.560	7.9	0.799	0.75	
23	H. G.	"	" 8	0.662	0.602	0.91	0.560	7.7	0.797	0.76	
24	H. R.	"	" 19	0.647	0.597	0.92	0.555	8.3	0.792	0.75	
25	K. K.	"	" 23	0.659	0.620	0.94	0.565	7.8	0.795	0.78	
26	J. T.	"	" 27	0.666	0.602	0.90	0.560	7.7	0.803	0.75	
27	J. D.	"	Mar. 1	0.644	0.589	0.91	0.560	8.2	0.787	0.75	
28	S. G.	"	" 2	0.640	0.588	0.92	0.565	8.8	0.794	0.74	
29	H. B.	"	" 5	0.662	0.610	0.92	0.565	8.3	0.810	0.75	
30	H. L.	"	" 5	0.659	0.598	0.91	0.570	8.1	0.803	0.75	
Maximum.....				0.674	0.629	0.94	0.570	8.8	0.811	0.78	
Minimum.....				0.640	0.588	0.90	0.555	7.7	0.787	0.74	
Average.....				0.658	0.604	0.917	0.562	8.05	0.799	0.755	
No. of determinations ...				30	10	10	10	10	10	10	10

\* Grams of NaCl per 100 cc., therefore, possibly slightly lower than previous figures after Bang's method (grams of NaCl per 100 gm.)

to fall along the diagonal line in both normal and pathological cases. It having been established that the changes in conductivity found in various diseases is principally due to a change in the salt content, it remains to be discovered why these changes occur. The nature of several of the groups showing decreased

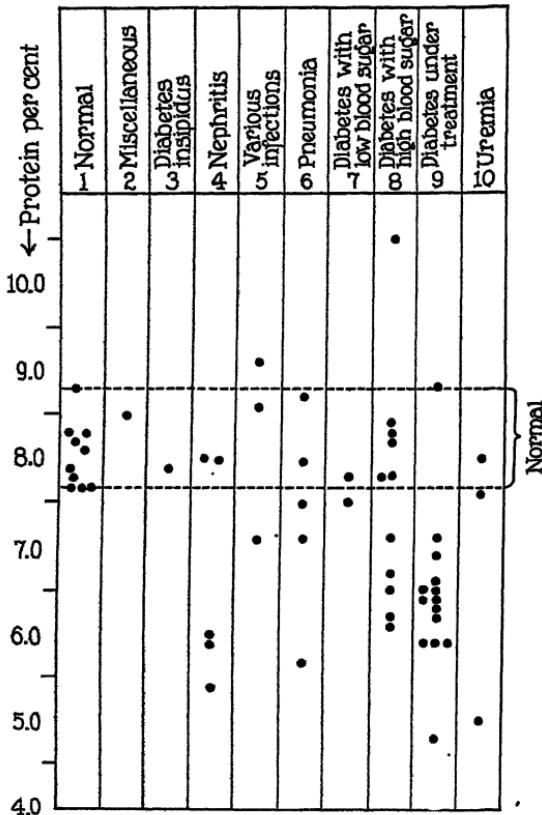


FIG. 4. Protein concentration in various sera.

conductivities made it probable that a key to the question might be obtained by determining the freezing point of the sera, which gives an expression of the osmotic pressure or total content of dissolved matter in the serum. This was done on a series of cases and the results are to be found in Fig. 7 and in the tables. The normal freezing point depression varied between 0.570 and

0.555, with an average of 0.562°C. A pronounced tendency to increase the depression was found in uremia and in the diabetics with high blood sugar, while the pneumonias nearly all showed a

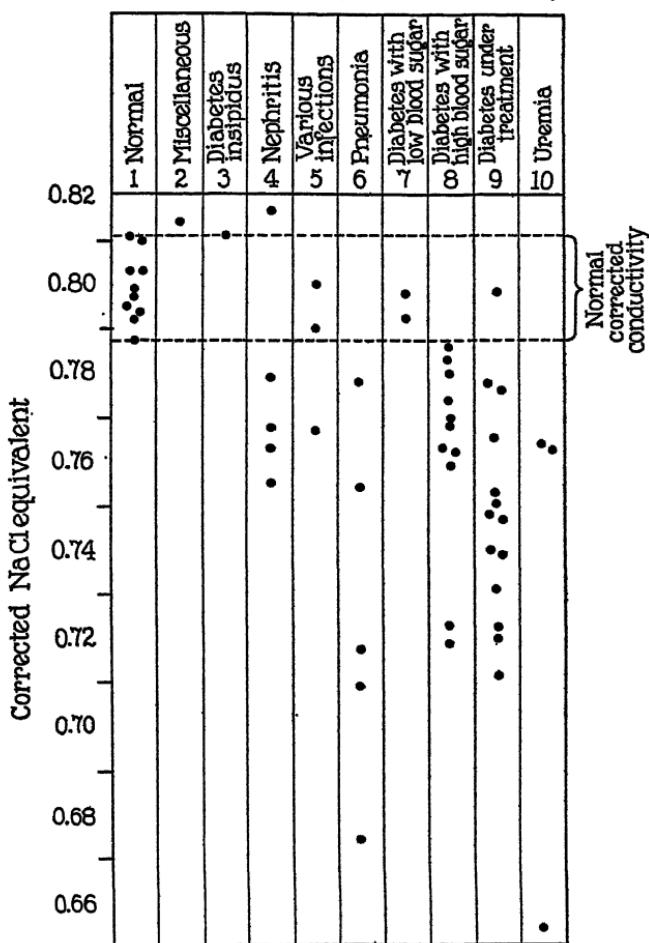


FIG. 5. NaCl equivalents of conductivity in various sera corrected for protein influence.

pronounced decrease in the depression (hence in the osmotic pressure). In nephritis one case showed increased depression, one normal, and several diminished. The prevalence of the

TABLE III.  
*Miscellaneous Cases.*

No.	Sex.	Date.	NaCl equivalent of conductivity.	NaCl percentage (titrated).	Ratio: NaCl percentage / NaCl equivalent.	$\Delta -^{\circ}\text{C}$ (cryoscope).	Protein percentage (refractometer).	NaCl equivalent corrected for protein influence.	Ratio: NaCl percentage / Corrected NaCl equivalent.	Diagnosis.
72*	M.	Dec. 20	0.659	0.615	0.93	0.560	0.570	0.814	0.76	Epilepsia. Dyspepsia.
73	"	" 29	0.644	0.600	0.93					
74	"	Jan. 26	0.662	0.617	0.93					Gastric ulcer.
Maximum.....			0.670	0.617	0.93	0.570				
Minimum.....			0.632	0.600	0.93	0.560				
Average.....			0.655	0.611	0.93	0.565	8.5	0.814	0.76	
No. of determinations.....			76	3	3	2	1	1	1	
Above normal.....			0	0	0	0	0	1	0	
Normal.....			75	3	3	2	1	0	1	
Below normal.....			1	0	0	0	0	0	0	

\* 73 specimens from 71 cases, on which only ionometer readings were taken, are not tabulated. They comprise diseases of the joints, muscles, nerves, brain, stomach, intestines, heart, blood, and endocrine glands. Among these there was only one showing conductivity below the normal; i.e., a case of colitis (0.632 NaCl equivalent). Numerous other cases of this disease were normal in this respect.

decreases in this group, however, is due to the fact that edematous cases were studied during the period in which osmotic pressure was determined. If we plot the results with conductivity as ordinate and freezing point as abscissa we get the graph presented in Fig. 8 in which the outlined rectangular area represents the normal range of variations. In this area will be found the

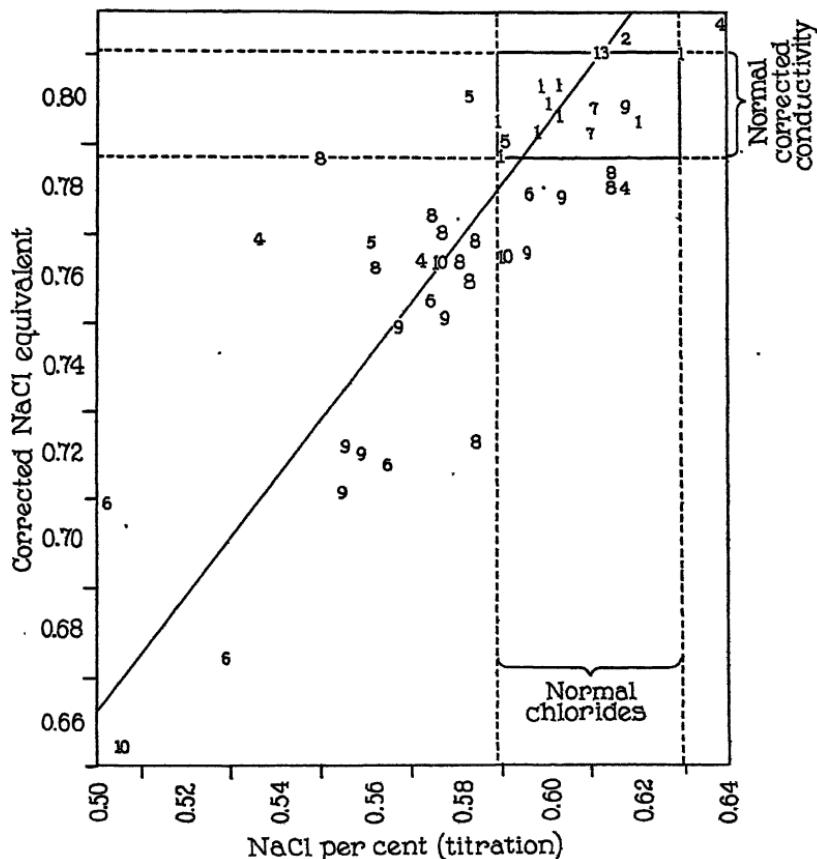


FIG. 6. Relation between corrected NaCl equivalent and titrated NaCl in various sera. The dotted lines represent the normal boundaries. The diagonal line represents the average normal ratio

$$\frac{\text{NaCl titrated}}{\text{Corrected NaCl equivalent}} = 0.755$$

TABLE IV.  
*Diabetes insipidus.*

No.	Name.	Sex.	Date.	NaCl equivalent of conductivity.	NaCl percent- age (titrated).	Ratio: NaCl percent- age — NaCl equiva- lent	$\Delta - {}^{\circ}\text{C.}$ (cryo- scope).	Protein percen- tage (refrac- tometer).	NaCl equiva- lent corrected for protein influence.	Ratio: NaCl percent- age — Corrected NaCl equiva- lent.	Remarks.
1	M. V.	F.	Feb. 20	0.670	0.612	0.91	0.565	7.9	0.811	0.75	Treated with pituitrin.

All values normal.

ten normal cases, two indifferent cases, a case of diabetes insipidus, the low blood sugar diabetics, and some infections and treated diabetics.

In the left lower area the pneumonias excepting one and a few of the nephritis cases are found, indicating a decrease of conductivity with decrease of osmotic pressure.

In the right lower area we find most of the diabetics with high blood sugar, most of the uremias, and a few of the treated diabetics

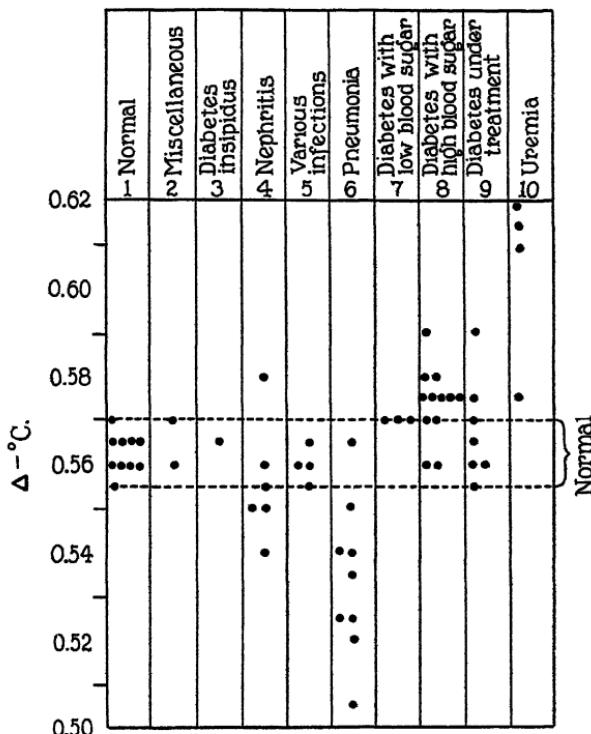


FIG. 7. Freezing point depression ( $\Delta - ^\circ\text{C}.$ ) in various sera.

that exhibit increased osmotic pressure with decrease of conductivity. However, some of the diabetics show decrease of conductivity with normal osmotic pressure (lower middle area) and a few normal or increased (observed) conductivity with increased osmotic pressure. One case of uremia also falls in the

latter area. A few nephritic cases are scattered in the upper middle and right areas indicating increase of conductivity with normal or slight increase of osmotic pressure. Another graph—Fig. 9—is plotted in the same way as Fig. 8, but using the cor-

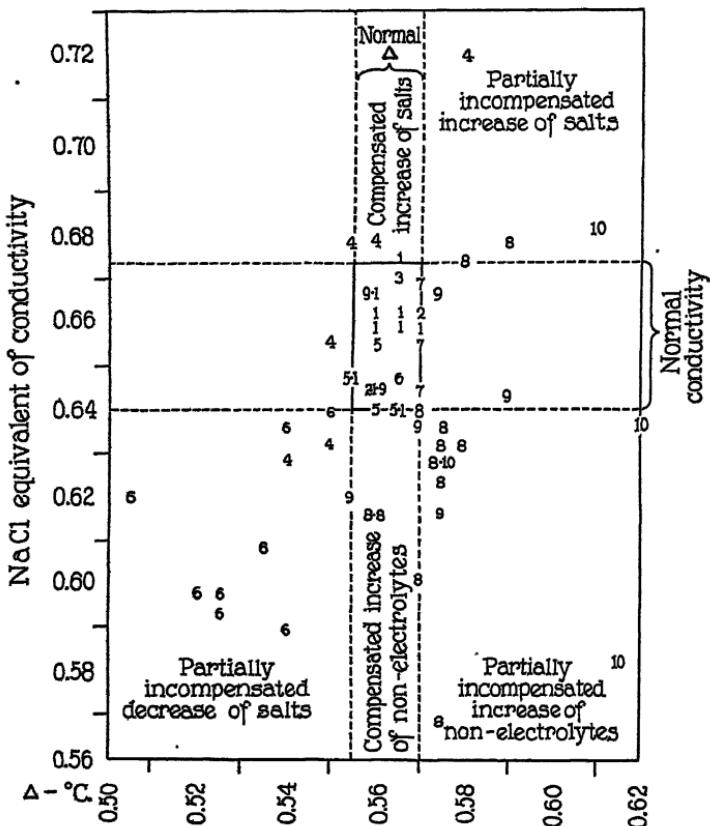


FIG. 8. Relation between NaCl equivalent of conductivity (ordinate) and freezing point (abscissa) in various sera. The dotted lines represent the normal boundaries.

rected NaCl equivalents of conductivity. On the whole the results using the corrected values are much the same but somewhat more consistent, since the high uncorrected conductivities in the diabetes and uremia are brought down to a low level by the

correction, the increase having been due to protein decrease and not to an increase of salts. Unfortunately, data were not obtained for this purpose on some of the cases of nephritis with high conductivity as they had been observed early in the series.<sup>6</sup>

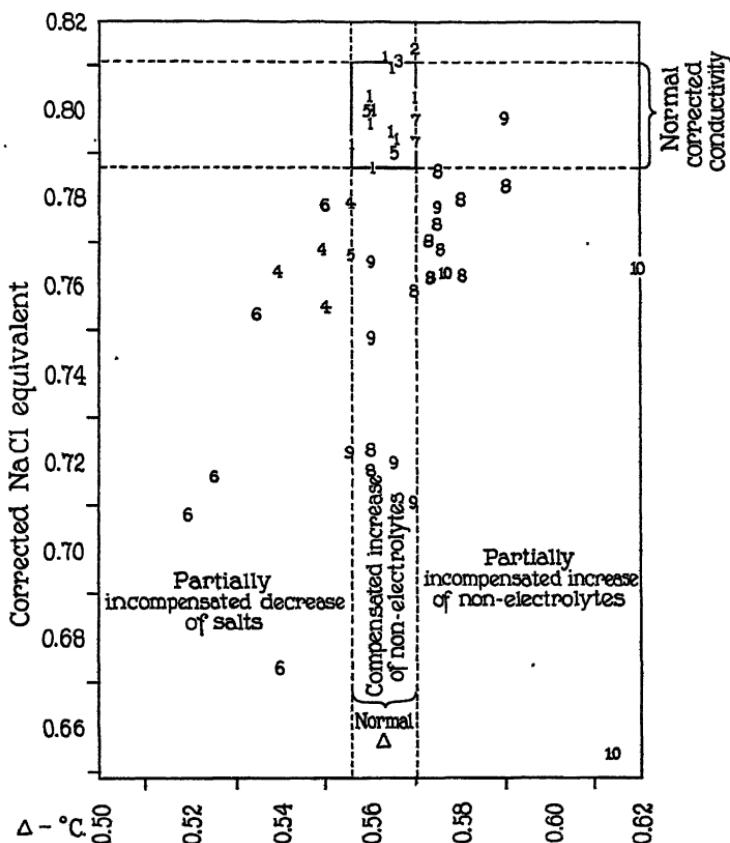


FIG. 9. Relation between corrected NaCl equivalent of conductivity (ordinate) and freezing point (abscissa) in various sera. The dotted lines represent the normal boundaries.

<sup>6</sup>This might have been of great interest, as the chlorides do not quite follow the upward movement of conductivity. In one case (Table V, No. 16) the discrepancy between apparent conductivity and chlorides is evidently due to a low protein percentage of the serum, as it disappears after correction.

*Nephritis, Simple Arterial Hypertension, and Edema Due to Other Causes (Uremia Cases Excluded).*

No.	Name.	Sex.	Date.	NaCl equivalent of conductivity.	NaCl percent-age (titrated).	Ratio: NaCl percent-age / NaCl equivalent.	$\Delta - {}^{\circ}\text{C}$ . (Cryoscopy).	Protein percent-age (refractometer).	NaCl equivalent corrected for protein influence.	Ratio: NaCl percent-age / Corrected NaCl equivalent.	Remarks.
1	T. K.	M.	Aug. 6	0.648							Nephrosclerosis. Blood pressure 240.
2	T. C.	"	" 2	0.661							Chronic nephritis. Blood pressure 165.
3	K. E.	F.	Sept. 19	0.653							Slight nephritis. Blood pressure 120.
4	A. J.	"	" 19	0.674							Arterial hypertension. Blood pressure 165.
5	E. F.	"	" 20	0.657							Arterial hypertension. Blood pressure 240.
6	A. A.	"	" 21	0.687							Chronic nephritis. Blood pressure 150.
7	M. E.	"	" 21	0.670							Chronic nephritis. Blood pressure 190.
8	V. A.	M.	June 10	0.653							Subacute nephritis. Blood pressure 130.
9	A. A.	"	" Aug. 21	0.670							Chronic nephritis.
			" 22	0.657							



TABLE VI.  
*Various Infections Excluding Pneumonia.*

No.	Name.	Sex.	Date.	NaCl equivalent of conductivity.	NaCl percentage (infrared).	Ratio: NaCl percentage (NaCl equivalent).	$\Delta - {}^{\circ}\text{C}$ .	Protein percentage (refractometer).	NaCl corrected for protein influence.	Ratio: NaCl percentage (NaCl equivalent).	Correced NaCl equivalent.	Remarks.
1	L. S.	F.	Sept. 4	0.620								Granulomatosis. High fever.
2	S. B.	"	" 4	0.632								" "
3	A. F.	M.	" 6	0.661								Renal tuberculosis. Afebrile.
4	C. A.	F.	" 7	0.653								Pleurisy. Subfebrile.
5	S. R.	M.	" 8	0.661								Rheumatic fever. High fever.
6	H. A.	"	" 11	0.620								Sepsis. High fever.
7	A. L.	F.	" 23	0.635								Pyelocystitis. Moderate fever.
8	K. A.	"	" 23	0.620								Tubercular meningitis. High fever.
9	M. H.	M.	" 26	0.661								Pulmonary tuberculosis. Light case.
10	A. V.	"	" 27	0.657								Pulmonary tuberculosis. Light case.
11	A. B.	F.	" 28	0.644								Dry pleurisy. Subfebrile.
12	H. W.	"	" 28	0.648								Pulmonary tuberculosis. Light case.
13	A. C.	"	" 28	0.628								Salpingitis. Afebrile.
14	E. N.	M.	" 28	0.648								Pulmonary tuberculosis. Light case.
15	O.I.	"	Dec. 14	0.636								Pulmonary tuberculosis. Hemiparesis. Unconscious. Febrile.

	J. G.	M.	Jan.	2	0.655	0.604	0.92	0.560	8.6	0.790	0.75	Influenza.	Subfebrile.	
16	J. G.	M.	"	17	0.640	0.589	0.92	0.565	9.1	0.800	0.73	Cerebrospinal syphilis.	No fever.	
17	C. J.	F.	"	18	0.640	0.582	0.91	0.560	7.1	0.767	0.73	Rheumatic fever.	High fever.	
18	P. G.	F.	"	19	0.647	0.560	0.87	0.555	7.1	0.767	0.73	Influenza.	High fever.	
19	W. N.	M.												
Maximum .....					0.661	0.604	0.92	0.565	9.1	0.800	0.75			
Minimum .....					0.620	0.560	0.87	0.555	7.1	0.767	0.73			
Average .....					0.642	0.584	0.905	0.560	8.8	0.786	0.787			
No. of determinations .....					19	4	4	4	4	3	3			
Above normal .....					0	0	0	0	1	0	0			
Normal .....					12	2	3	4	1	2	1			
Below normal .....					7	2	1	0	1	1	2			

TABLE VII.  
*Pneumonia.*

No.	Name.	Sex.	Date.	NaCl equivalent of conductivity.	Ratio NaCl per cent age (titrated).	$\Delta - ^\circ\text{C}$ .	NaCl equivalent corrected for protein influence.	Ratio: NaCl percent age.	Current NaCl equivalent.	Remarks.
1	N. H.	M.	Sept. 8	0.635						High fever. " " "
2	C. M.	"	29	0.632						III 4 days. High fever. " " "
3	O'Co.	"	Dec. 9	0.628						Ill few days. " " " " " "
4	C. Wa.	"	" 9	0.613						" " " "
5	W. McN.	"	" 15	0.613						" " " "
6	N. N.	"	" 22	0.640						" " " "
7	A. B.	"	Jan. 2	0.594	0.517	0.87	0.525			At crisis.
8	C. W.	"	" 11	0.647	0.570	0.88	0.565			III 6 days. High fever. " a week. " "
9	W. G.	"	" 12	0.636	0.543	0.85	0.540			" few days. " "
10	C. Wi.	"	" 13	0.620	0.532	0.86	0.505			" few days. " "
11	E. H.	F.	" 16	0.598	0.502	0.84	0.520	7.1	0.700	5 days. " "
12	N. S.	M.	" 24	0.609	0.574	0.94	0.535	8.7	0.754	2 " "
13	T. G.	"	" 29	0.590	0.528	0.90	0.540	5.7	0.674	few days. " "
14	S. A.	F.	Feb. 6	0.640	0.595	0.93	0.550	8.0	0.778	3 days. Moderate fever. " 4 " " "
15	L. W.	M.	" 16	0.598	0.564	0.94	0.525	7.5	0.717	0.79
Maximum .....				0.647	0.595	0.94	0.565	8.7	0.778	0.79
Minimum .....				0.590	0.502	0.84	0.505	5.7	0.674	0.71
Average .....				0.620	0.547	0.890	0.534	7.4	0.726	0.780

## Diabetes with Low Blood Sugar.

No.	Name.	Sex.	Date.	NaCl-equivalent of conductivity.	NaCl-parentage (titrated).	Ratio: NaCl-parentage / NaCl-equivalent.	$\Delta - ^\circ\text{C}$ .	Protein percentage (refractometer).	NaCl-equivalent corrected for protein influence.	Ratio: NaCl-parentage / NaCl-equivalent.	Corrected NaCl-equivalent.	Remarks.
1	M. P.	F.	Sept. 1	1928	0.670							- Acidosis. Blood sugar 0.120.
2	V. N.	M.	" 11	0.644								- " " 0.080.
3	M. N.	"	" 12	0.648								- " " 0.124.
4	K. H.	F.	" 12	0.666								Urine - sugar. " -
5	M. P.	"	" 12	0.644								- Acidosis. Blood sugar 0.092.
6	M. K.	"	" 14	0.645								Urine - sugar.
7	J. R.	"	" 19	0.657								Blood sugar 0.076.
8	C. O.	"	Dec. 13	0.681								" " 0.087.
9	G. S.	M.	" 22	0.644	0.596	0.93	0.570					- Acidosis. Blood sugar 0.147.
10	Ooo.	F.	Mar. 1	0.666	0.610	0.92	0.570	7.5	0.798	0.76		" " 0.122.
11	W. E.	M.	" 3	0.655	0.609	0.93	0.570	7.8	0.792	0.77		
<b>Maximum.....</b>				0.670	0.610	0.93	0.570	7.8	0.798	0.76		
<b>Minimum.....</b>				0.644	0.596	0.92	0.570	7.5	0.792	0.77		
<b>Average.....</b>				0.656	0.605	0.927	0.570	7.65	0.795	0.765		
<b>No. of determinations.....</b>				11	3	3	3	2	2	2		
<b>Above normal.....</b>				1	0	0	0	0	0	0		
<b>Normal.....</b>				10	3	3	3	1	2	2		
<b>Below normal.....</b>				0	0	0	0	1	0	0		





TABLE X.  
*Diabetes under Treatment.\**

No.	Name.	Sex.	Date.	NaCl equivalent of conductivity.	NaCl percent-age (titrated).	Ratio: NaCl percent-age / NaCl equivalent.	$\Delta - ^{\circ}\text{C}$ (crys- toscopes).	Protein percent-age (refrac- tometer).	NaCl equivalent corrected for protein influence.	Ratio: NaCl percent-age / NaCl equivalent.	Remarks.
1	M. P.	F.	Sept. 12 a.m.	1928	0.644						-Sugar in urine. Received for other purposes 50 gm. glucose in the morning.
2	W. C.	M.				p.m.	0.628				+Sugar in urine.
3	H. H.	"	Aug. 28	0.608	0.600	"	0.635				+Acidosis. Blood sugar 0.275.
4	H. J.	"	Sept. 5	0.635	0.620	"	0.620			" " 0.241.	
			"	0.635	0.640	"	0.640			" " 0.271.	
5	L. H.	"	"	0.620	0.620	"	0.620			" " 0.232.	
6	M. T.	F.	Dec. 1	0.647	0.635	"	0.635			Slight acidosis. Blood sugar 0.159.	
			"	0.647	0.640	"	0.640			Slight acidosis. Blood sugar 0.136.	
7	A. H.	M.	Jan. 22	0.674	0.666	"	0.666			+Acidosis. Blood sugar 0.158.	
			"	0.674	0.668	"	0.668			" " 0.169.	
			"	0.674	0.668	"	0.668			Slight acidosis. Blood sugar 0.214.	
			"	0.674	0.668	"	0.668			Acidosis decreased. Blood sugar 0.198.	
			"	0.674	0.668	"	0.668			Complicating nephritis. Blood sugar 0.228.	
			"	0.674	0.668	"	0.668			No material change.	

8	T. C.	M.	Feb. 12	0.616	0.584	0.95	0.560	6.7	0.723	0.81	++ Acidosis.	Blood sugar 0.327.
			" 14	0.644				5.9	0.740		Less acidosis.	Blood sugar 0.153.
			" 15	0.640				6.5	0.747		Less acidosis.	Blood sugar 0.190.
			" 19	0.670				6.2	0.776		No acidosis.	Blood sugar 0.150.
			" 6	0.644	0.617	0.96	0.590	8.8	0.798	0.77	No acidosis.	Blood sugar 0.225.
9	Fr.	F.	" 2	0.624	0.561	0.90	0.575	8.2	0.762	0.74	+ Acidosis.	High blood sugar on previous days.
			" 14	0.624				7.1	0.739		+ Acidosis.	Blood sugar 0.227 (Feb. 15).
			" 16	0.620				6.9	0.731		Slight acidosis.	Blood sugar 0.188.
			" 24	0.644	0.577	0.90		6.4	0.790	0.77	Slight acidosis.	Blood sugar 0.200.
			Mar. 2	0.644	0.567	0.88	0.560	6.3	0.748	0.76	Slight acidosis.	Blood sugar 0.240.
10	S. S.		Feb. 1	0.678	0.614	0.91	0.590	6.1	0.783	0.78	Still high blood sugar.	Blood sugar 0.177.
			" 19	0.655				5.9	0.753		+ Acidosis.	Blood sugar 0.214.
			" 26	0.666	0.803	0.91	0.575	6.5	0.777	0.78	"	" 0.206.
			" 24	0.616			0.560	6.5	0.719		"	" 0.142.
			" 26	0.616	0.559	0.91	0.565	6.6	0.720	0.78	"	" 0.234.
11	W. R.	M.	" 27	0.620	0.556	0.90	0.555	6.4	0.722	0.77	"	" 0.133.
			Mar. 1	0.636	0.556	0.87	0.570	4.8	0.711	0.78	"	" 0.133.
			" 5	0.666	0.595	0.89	0.560	5.9	0.765	0.78		

\* The first examinations of the patients in this table are also found in the two previous tables. The reasons for creating this group are situated in foot-note 5.

TABLE XI.  
*Uremia.*

No.	Name.	Sex.	Date.	NaCl-equivalent of conductivity.	NaCl-percentage (titrated).	$\Delta^{\circ}\text{C}$ . (cryoscope).	Protein-percentage (refractometer).	NaCl-equivalent corrected for protein influence.	Ratio: NaCl percentage / Corrected NaCl equivalent.	Remarks.	
1	J. J.	M.	May 5 1928	0.597	0.597					Non-protein N 0.094.	
2	Ea.	"	Nov. 28 Dec. 2 " 13	0.628 0.598 0.624	0.532 0.538	0.89 0.86				Blood urea, N 0.108. " " 0.158. " " 0.160.	
3	Ma.	"	1928								
4	W. J.	"	Jan. 6 " 18 Feb. 10 Mar. 14	0.681 0.582 0.628 0.636	0.649 0.505 0.574 0.590	0.95 0.87 0.91 0.93	0.610 0.615 0.575 0.620	5.0 8.0 8.0 7.6	0.654 0.77 0.763 0.764	" " 0.120. " " 0.172. " " 0.077. " " 0.127 (2 days earlier).	
5	A. O'N.	F.									
6	S. P.	"									
7	Hn.	M.									
Maximum.....		0.681		0.649		0.95		0.620		8.0	
Minimum.....		0.582		0.505		0.86		0.575		5.0	
Average.....		0.622		0.565		0.902		0.605		6.9	
No. of determinations.....		8		6		6		4		3	
Above normal.....		1		1		1		4		0	
Normal.....		0		1		2		0		0	
Below normal.....		7		4		3		0		3	

TABLE XII.  
*Jaundice.*

No.	Name.	Sex.	Date.	NaCl equivalent percent-age (dissolved conductivity concentrated).	NaCl percent-age (titrated).	Ratio: NaCl percent-age / NaCl equivalent.	$\Delta - ^{\circ}\text{C}$ . (cryoscopie).	Protein percent-age (refractometer).	NaCl equivalent corrected for protein influence.	Ratio: NaCl percent-age / NaCl equivalent.	Remarks.
1	A. N.	F.	Aug. 21	0.632							
2	S. S.	"	Sept. 9	0.644							
3	V. P.	M.	" 16	0.635							
4	N. N.	"	" 10	0.648							
			" 22	0.625							
<i>1922</i>											
1	A. N.	F.	Aug. 21	0.632							
2	S. S.	"	Sept. 9	0.644							
3	V. P.	M.	" 16	0.635							
4	N. N.	"	" 10	0.648							
			" 22	0.625							
<i>1922</i>											
Maximum.....						0.648					
Minimum.....						0.625					
Average.....						0.637					
No. of determinations.....						5					
Above normal.....						0					
Normal.....						2					
Below normal.....						3					

\* Meulengracht method. Normal values 1 to 5.

The graphs do not show the individual peculiarities of the cases nor the concentration of urea, sugar, or ketones in the blood; for this purpose it will be necessary to consult the "Remarks" column in the tables. The lack of quantitative estimation of blood ketones and other non-protein nitrogen than that derived from urea, of course, makes it impossible to make direct comparisons between the amount of non-electrolytes and the decrease in conductivity. In consideration of these findings one may conclude that the decrease in conductivity found in pneumonia is due directly to a primary decrease of salts. Such a deficiency of salts is suggested by the well known fall in chloride elimination in pneumonia. It is possible that a diet poor in salts and the formation of large exudates are factors in this chloride deficiency. The importance of these factors is also suggested by the fact that some nephritis with edema on a low chloride diet showed low salt content and low osmotic pressure. A very slight compensation of the osmotic pressure may be effected by the small increases in blood urea frequently found in pneumonia; these changes, however, are far too small to bring the osmotic pressure back to normal.

In diabetes with high blood sugar (and ketonemia), in uremia (and presumably also in jaundice), the decrease in conductivity (*i.e.*, salts) represents a sort of regulatory mechanism, which tends to keep down the increased osmotic pressure. If this regulation fails—as it will in some instances—the osmotic pressure will go up higher than in a regulated case with the same amount of non-electrolytes. The difficulty of estimating more than one or two of the non-electrolyte factors make this more or less difficult to show conclusively in all cases. As to the nature of this process there are no data. It might be conceived as being a function of the tissues since it can be observed even with extreme renal insufficiency when regulation by variation in composition of the urine is impossible.

Incidentally, the results put forward in this paper would seem to indicate administration of salt in pneumonia and institution of a diet poor in salts in severe uremia in order to facilitate osmotic regulation.

SUMMARY.

1. The NaCl equivalent of the serum conductivity at 20°C. varies between 0.674 and 0.640 per cent. In nephritis high, low, and normal values are found. Most pneumonias and some other infections, most uremias, and diabetics with high blood sugar show a low conductivity. The rest of the diseases, including diabetes with low blood sugar, shows a normal conductivity.

2. There is a marked constancy in the ratio between the titrated NaCl concentration and the NaCl equivalent of conductivity of sera, the average normal ratio being 0.917. The normal concentration of chlorides in this series varied between 0.629 and 0.588, with an average of 0.604 per cent of NaCl.

3. The protein percentage of normal serum varies between 7.7 and 8.8 per cent, with an average of 8.05 per cent. In various diseases large variations are found. If we correct the conductivity values found by the depression caused by the protein percentage of the serum, we get a corrected sodium chloride equivalent, which in normals varies between 0.811 and 0.787 per cent, with an average of 0.799 per cent. The trend of variations in the pathological groups is even clearer when these corrected values are used. The average normal ratio between NaCl and the corrected NaCl equivalent of the conductivity is 0.755 and this ratio tends to be slightly closer to the normal in the pathological cases, than that derived from the uncorrected conductivity.

4. The freezing point of normal sera varies between -0.570 and -0.555, the average being -0.562°C. Low values of osmotic pressure are found in pneumonia and some other infections, while high values occur frequently in diabetes (with high blood sugar) and uremia. In diabetes with normal blood sugar the osmotic pressure is normal. In nephritis both high and low osmotic values are found.

5. The relationship between conductivity and osmotic pressure appears to be as follows:

(a). Decreases of conductivity are due to changes in the salt content of the serum, a fact which appears even more clearly when the variations due to different protein concentrations are taken into consideration. This decrease occurs under two circumstances: (1) A primary decrease of salts with consequent

low osmotic pressure in cases with simultaneous low salt intake and formation of exudates or transudates. Type: Pneumonia and edematous cases on salt-free diet. (2) A secondary decrease of salts tending to keep down the high osmotic pressure caused by an increase of non-electrolytes (sugar, urea, gall constituents). Type: Diabetes with high blood sugar, uremia, and presumably also jaundice.

(b). A true increase of conductivity (and chlorides) is found in some cases of nephritis. The number of such cases thoroughly examined is too small to allow any conclusions as to the origin or significance of the change; the increase of conductivity, however, may be due to a low protein content of the serum in which case it disappears on correction and is not accompanied by a correspondingly high chloride concentration. It may be added here that in no case was the protein concentration so increased that an abnormally low conductivity was obtained.

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## THE INITIAL ACIDOSIS IN ANESTHESIA.\*

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Investigations of the acid-base equilibrium of the blood have mainly concerned themselves; first, with the chemical mechanism for maintaining the constancy of reaction through the action of the buffer systems of the blood; and secondly, with the physiological studies which have shown the importance of the respiratory center and of the kidneys in promoting the same constancy of reaction. In the studies of disease striking instances of strain thrown upon this mechanism have come to light and have been described as various types of acidosis and of alkalosis. The normal range of the alkali reserve of man and certain species of animal has been fairly well established. Less well established is the normal range of pH of the blood.

On the other hand, we have but little information concerning the variation from minute to minute of the alkali reserve, the pH, the CO<sub>2</sub> tension, and the oxygen tension in the mixed arterial blood. Still less information is available as to the variation in these quantities in different vascular areas of the body. Few studies throw light upon the nature of the factors tending to alter these quantities from minute to minute.

In our studies upon the acidosis occurring in ether anesthesia we obtained evidence of extremely rapid fluctuations in the alkali reserve as well as in other factors concerned in the acid-base equilibrium.

Changes in the acid-base balance of the blood during ether anesthesia have been studied by many investigators. There has been some difference in the interpretation of the changes observed.

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In a previous paper Van Slyke, Austin, and Cullen reviewed this literature and reported studies upon the changes in  $\text{CO}_2$  content and hydrion concentration of the arterial serum of dogs during ether anesthesia. From these data the alkali reserve of the true serum was calculated. They defined the alkali reserve as the bicarbonate content of the true serum when the oxygenated blood is equilibrated with  $\text{CO}_2$  to give the same pH as that at the initial bleeding of the experiment. They found after ether anesthesia of from 20 to 60 minutes duration a decrease in the alkali reserve of from 6 to 15 mm. (13 to 34 volumes per cent) bicarbonate and an increase in the hydrion concentration of 0.2 pH or more. These results indicated a *primary* acidosis developing in association with an increased hydrion concentration of the blood.

The purpose of this report is to present further experiments upon the behavior of the acid-base balance of the blood during the early minutes of anesthesia and under certain other relevant conditions. The experiments were planned to study the rate at which changes in the acid-base equilibrium and especially in the alkali reserve may occur and to investigate some of the factors which might induce changes in the alkali reserve in the course of a few minutes. It will be shown that a large part of the fall in alkali reserve occurs in the first few minutes of anesthesia.

#### *Methods.*

*Animals.*—Large dogs (10 to 20 kilos) were used so that large samples of blood (25 to 120 cc.) might be taken. The dogs were fed as usual the day preceding the experiment, but received no food on the day of the experiment.

*Bleeding.*—The blood was drawn from the left ventricle (unless stated otherwise) through a 4 inch, 16 gauge lumbar puncture needle into tubes under oil. When oxalated blood was desired the tube was previously coated with neutral potassium oxalate to make 0.3 per cent. In centrifuging for true serum the blood was drawn directly into a centrifuge tube of the proper size, containing mineral oil. The glass delivery tube was withdrawn as the blood ran in, so that the tube was completely filled with the blood except for a layer of paraffin oil about 1 cm. deep. A 1-hole rubber stopper was inserted, with complete expulsion of the oil.

The hole was closed with a glass plug and the tube centrifuged at once. After centrifuging, the glass plug was removed, and from a pipette oil was allowed to flow through the hole in the stopper as the stopper was removed. It is shown elsewhere that such precautions are necessary to prevent loss of CO<sub>2</sub> during centrifuging (Austin and coworkers). The plasma or serum was then transferred without loss of CO<sub>2</sub> to Haldane sampling tubes over mercury or to tubes under oil.

When both oxalated blood and true serum were required from the same bleeding, the tubes were connected to the needle by a small Y-tube. The blood was then directed as desired by clamping one or the other connecting rubber tube. Usually the oxalated tube was filled last in order to permit of prompt stirring before coagulation set in.

*Anesthesia.*—The animals were anesthetized with chloroform by the drop method with a few layers of gauze or with ether by use of a towel saturated with ether until the animal relaxed and then with a few layers of gauze onto which the ether was dropped.

#### *Technique of Gas Administration.*

In the administration of the gases N<sub>2</sub>O, N<sub>2</sub>, and O<sub>2</sub> and in determining ventilation rate a mask was used. This mask was made from the inner tube of an automobile tire and was shaped like a truncated cone. The larger end of the cone fitted closely over the dog's muzzle, the smaller end was closed by a well greased No. 10 cork stopper. A short Y-tube of 1 inch glass tubing was inserted into a hole in the No. 10 stopper. One arm of the Y-tube led through an inlet valve (Siebe-Gorman) and 6 feet of 1 inch tubing to a spirometer, the other arm of the Y to an outlet valve.

The gas mixtures were introduced into the spirometer and during the administration the spirometer was forced down by hand so that there was always a brisk flow of gas either through the Y-tube, or in other experiments, through the entire mask.

*For ventilation rate measurement* the inlet and outlet valves were interchanged. The spirometer was balanced carefully to eliminate resistance.

*Hydrogen Ion Concentration Measurements.*

*Electrometric.*—The determinations were made at 38°C. on serum in the manner described elsewhere (Cullen, 1922).

*Colorimetric.*—The colorimetric pH measurements were made at room temperature with phenol red in diluted serum by Cullen's method (1922). The correction to reduce colorimetric pH at 20° to the true pH of undiluted serum at 38° was taken as -0.34 pH.

*Analytical Methods.*

The carbon dioxide determinations were made, usually in duplicate, by Van Slyke's method, using the constant volume apparatus. The oxygen determinations were made with the technique described by Van Slyke and Stadie, using the original Van Slyke apparatus.

*Calculation.*

In this report the following abbreviations have been used; mm. for millimolar;  $[CO_2]$ ,  $[BHCO_3]$ ,  $[H_2CO_3]$ , and  $[O_2]$  for mm. concentrations of total  $CO_2$ , bicarbonate,  $H_2CO_3$ , and oxygen;  $CO_2$  tension is given in millimeters of mercury (mm.);  $\frac{d[BHCO_3]}{dpH}$  for the ratio of the increment in  $[BHCO_3]$  to the increment in pH as the blood changes along its  $CO_2$  absorption curve;  $\frac{d[CO_2]}{d[O_2]}$  for the ratio of the increment in total  $[CO_2]$  of the true serum to the increment in the  $[O_2]$  of the whole blood as the oxygenation of the blood changed while the pH of the blood is kept constant (by suitable change in  $CO_2$  tension).

The data obtained by analysis includes the total  $[CO_2]$  and the colorimetric, and in some cases the electrometric, pH of the serum as drawn, and the oxygen capacity and content of the blood as drawn. In one experiment total  $[CO_2]$  and pH determinations were made upon serum equilibrated with measured tension of  $CO_2$  at 38°.

The calculations of  $[H_2CO_3]$  and  $[BHCO_3]$  from the determined total  $[CO_2]$  and pH at 38° of the serum as drawn were based upon Hasselbalch's equation  $pH = pK_1 + \log \frac{[BHCO_3]}{[H_2CO_3]}$  assuming a  $pK_1$  of 6.10.

The CO<sub>2</sub> tension was calculated from the [H<sub>2</sub>CO<sub>3</sub>] on the basis of Bohr's solubility coefficient for CO<sub>2</sub> in plasma of 0.541 at 38°.

$$\text{CO}_2 \text{ tension at } 38^\circ \text{ in mm.} = \frac{[\text{H}_2\text{CO}_3]}{0.0318}$$

The alkali reserve of the blood as defined by Van Slyke is the factor which determines the elevation of the CO<sub>2</sub> absorption curve of the oxygenated blood, or, conversely the elevation of the CO<sub>2</sub> absorption curve may be taken as a measure of the alkali reserve.

Since at constant pH, change in [BHCO<sub>3</sub>] of blood or true serum is the direct measure of the change in base free to neutralize acids other than H<sub>2</sub>CO<sub>3</sub>, Van Slyke (1921, b)<sup>1</sup> has recommended that the elevation of the CO<sub>2</sub> absorption curve be expressed in units of [BHCO<sub>3</sub>] at constant pH rather than at constant CO<sub>2</sub> tension. It should be remembered that the two following propositions are included in the definition of the alkali reserve. (1) The alkali reserve at all points along a given CO<sub>2</sub> absorption curve of the whole blood or true serum is the same. (2) The alkali reserve is not changed by oxygenation or reduction of the hemoglobin.

In these studies we have been interested in changes in the alkali reserve rather than in its absolute value at a standard pH. We have, therefore, as in the preceding paper (Van Slyke, Austin, and Cullen) evaluated the alkali reserve of the successive bleedings of each experiment as the [BHCO<sub>3</sub>] of the true serum which would result if each blood were oxygenated and equilibrated with CO<sub>2</sub> to give the pH of the initial bleeding. The method of extrapolation used to calculate this value is based on two premises. First, there is evidence that if the CO<sub>2</sub> absorption curve of blood be plotted as [BHCO<sub>3</sub>] against pH the experimental points approximate a straight line. (There is a slight experimental deviation from this line in some bloods, but for our purpose and over the range through which we are working the approximation is sufficiently exact.)<sup>2</sup> Secondly, the CO<sub>2</sub> absorption curves of dog's true serum that we have determined exhibit slopes of  $\frac{d[\text{BHCO}_3]}{dpH}$  lying between -20 and -28; i.e., a rise of 0.1 in pH is associated with a fall from 2.0 to 2.8 mm. [BHCO<sub>3</sub>].

<sup>1</sup> Van Slyke (1921, b), p. 169.

<sup>2</sup> Peters, J. P., Eisenman, A. J., and Bulger, H. A., *J. Biol. Chem.*, 1923, Iv, 709.

In these experiments we have corrected for oxygen unsaturation when necessary by assuming a ratio  $\frac{d[\text{CO}_2]}{d[\text{O}_2]} = -0.52$ ; i.e., a rise of 1 mm.  $\text{O}_2$  saturation causes 0.52 mm. decrease in  $[\text{CO}_2]$ . This value was taken from the observations of Van Slyke, Hastings, and Neill who found values ranging from -0.50 to -0.59. Quite as suitable would have been the factor obtained by Doisy, Briggs, Eaton, and Chambers of -0.44. In the studies of Van Slyke, Hastings, and Neill the  $[\text{CO}_2]$  represents whole blood total  $\text{CO}_2$  whereas we are dealing with true serum, but the error which this introduces may be considered negligible for our purposes.

The value for  $[\text{BHCO}_3]$  after correction for oxygen unsaturation, if necessary, was extrapolated by using our limiting  $\frac{d[\text{BHCO}_3]}{dpH}$  ratios to the pH of the initial bleeding and the difference between the values thus calculated and the  $[\text{BHCO}_3]$  of the true serum at the initial bleeding was taken as the change in alkali reserve. These changes in  $[\text{BHCO}_3]$  at initial pH have been plotted as areas bounded by the values obtained by the use of the two limiting slopes.

*Presentation of Data.*—In all cases the charts are constructed to show the changes in the alkali reserve, pH,  $\text{CO}_2$  tension, and total  $[\text{CO}_2]$  as ordinates and with time as abscissæ. The absolute values for these quantities as determined or calculated are given in the tables.

#### EXPERIMENTAL.

The experiments presented in this paper may be considered in four groups. The first group includes those in which we studied the immediate effect of administration of ether or chloroform upon the alkali reserve and pH of the serum. The second group includes various control experiments designed to test the effect of repeated bleedings, exertion, and psychic disturbance of a degree comparable with that incidental to the experiments of the other groups. In this group are included also experiments designed to test the effect of the application to the dog's muzzle of a mask fitted with valves and connected with a spirometer for the measurement of ventilation rate and also the effect of increasing the dead space, of lowered oxygen tension, and of increased  $\text{CO}_2$  tension in the

inspired air. All these factors entered to some extent into the experiments of the other groups, but with one exception to no greater extent than in these control experiments.

The exception mentioned is the extremely low oxygen tension incidental to the use of nitrous oxide and this factor was controlled by an experiment reported in the third group.

The third group of experiments includes those in which we studied the immediate effects on the alkali reserve and pH of the serum of inhalation of pure nitrous oxide for 1 or 2 minutes followed by 95 per cent nitrous oxide and 5 per cent oxygen. It was found necessary with the dog to employ these high concentrations of nitrous oxide in order to secure and maintain anesthesia. Because of the marked anoxemia produced, similar experiments were performed in which nitrogen was substituted for the nitrous oxide.

As the fourth group (Experiments 18, 19, and 20), in view of the fact that the calculation in our experiments is dependent upon the measured pH of the serum, experiments were carried out to determine if any changes occurred in the blood after anesthesia or coincident with a fall in alkali reserve which changed either the  $pK_1$  of the serum or changed the constancy of relation of the colorimetric determination to the electrometric determination of hydrogen ion concentration.

In Experiment 18 it is evident that the  $pK_1$  of the serum was the same before and after anesthesia. In Experiments 19 and 20 it is evident that the decrease in pH as measured electrometrically is practically identical with the decrease in pH as determined colorimetrically. These results indicate the accuracy of the colorimetric pH measurement in experiments of this nature.

#### *Group I.*

(*Experiments 1, 2, 3, 4, 5, and 17; Figs. 1 and 2.*)

In the experiments of this group it will be observed that both with ether and with chloroform an extremely rapid fall in the alkali reserve of from 4 to 11 mm. occurs in the first few minutes. This is associated with rise of  $CO_2$  tension and fall of pH. As the anesthesia proceeds the fall in alkali reserve continues, but at continually decreasing rate. The  $CO_2$  tension tends to return

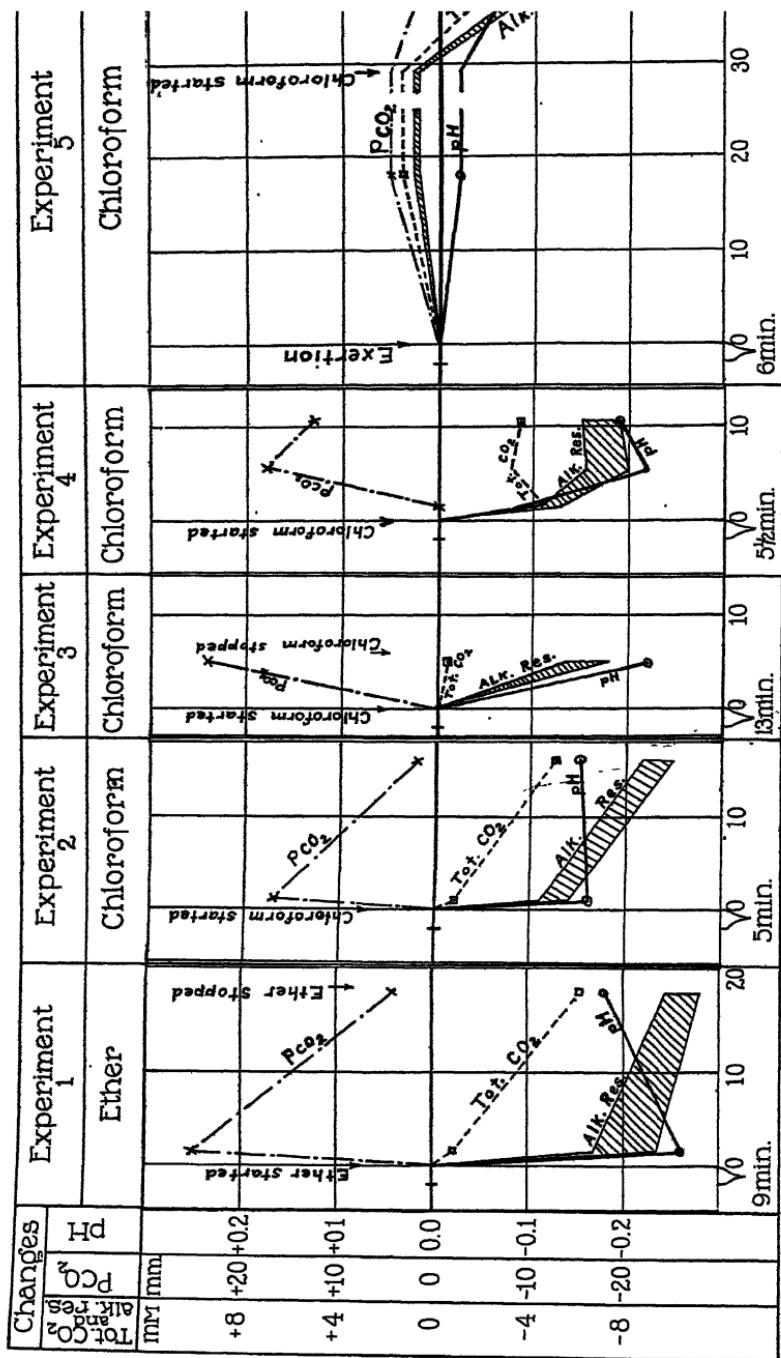


FIG. 1.

toward normal in consequence in part no doubt of the increasing ventilation rate which we reported in the previous study. Synchronously, either the rate of fall in pH diminishes or there may be a return toward normal pH. After cessation of ether anesthesia

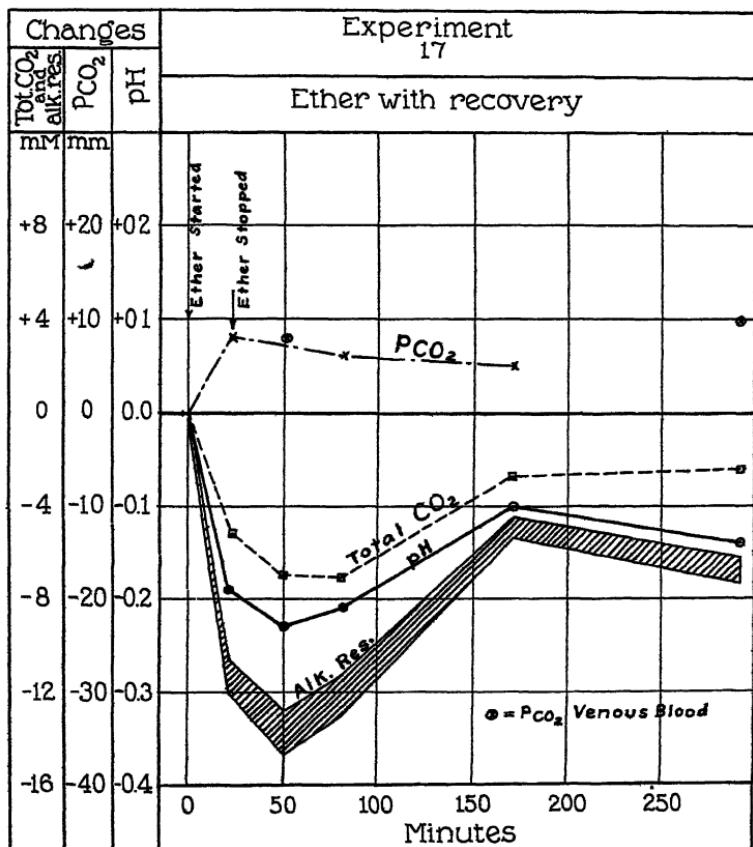


FIG. 2.

the return of the alkali reserve to normal commences almost immediately and in an experiment in which the fall had amounted to 14 mM. it had returned 9 mM. toward normal in  $2\frac{1}{2}$  hours, after which the return became less rapid. The CO<sub>2</sub> tension remained relatively constant during this return toward normal, the rise in

pH roughly paralleling the rise in alkali reserve. This tendency to constancy of CO<sub>2</sub> tension with parallel change in alkali reserve and pH has been noted in a considerable number of our experiments, but is entirely absent in others.

The rapidity of the fall of alkali reserve in the first few minutes of ether and chloroform anesthesia and its diminishing rate of fall as the anesthesia proceeds and becomes deeper suggests that this phenomenon is not a consequence entirely of altered cell metabolism due to narcosis *per se*. The facts suggest that the phenomenon might be a consequence of some of the incidental factors occurring during the induction of the anesthesia. However, the control experiments of Group II, inducing sometimes a rise and sometimes a fall of alkali reserve and never a change so marked as that observed in these experiments, seem to exclude at least individually the factors studied in those experiments. Explanation of the cause of the rapid fall in alkali reserve must await further studies: whether it is due to a withdrawal of base from or an introduction of acid into the blood; if the latter, the nature of the acid introduced; or whether vasomotor changes with redistribution of the proportion of the blood coming from different vascular areas have occurred. Indeed the significance of the acidosis of anesthesia would seem to rest upon a proper interpretation of this early fall in alkali reserve and until we can interpret this initial fall it is even impossible to state whether this acidosis is to be considered detrimental to the organism or rather a compensatory mechanism.

We would take the occasion to point out that in all of our experiments with ether and chloroform there is an increased oxygen unsaturation of the arterial blood during and immediately after the close of the anesthesia. The existence of a true acidosis at the end of the anesthesia does not in our opinion necessarily contraindicate the therapeutic use of inhalation of CO<sub>2</sub> to stimulate the respiratory center, although increased CO<sub>2</sub> tension would still further lower the pH. A greater ventilation of the lungs may be expected to lead to a more rapid removal of the anesthetic and to a more thorough oxygenation of the blood, both of which may be of greater benefit than the further lowering of the pH of the blood would be detrimental.

## Group II.

(Experiments 5, 9, 10, 12, 13, 14, 15, and 16; Figs. 3, 4, 5, and 6.)

The experiments of this group are described in detail in the respective protocols and the nature of each experiment is indicated in the following table:

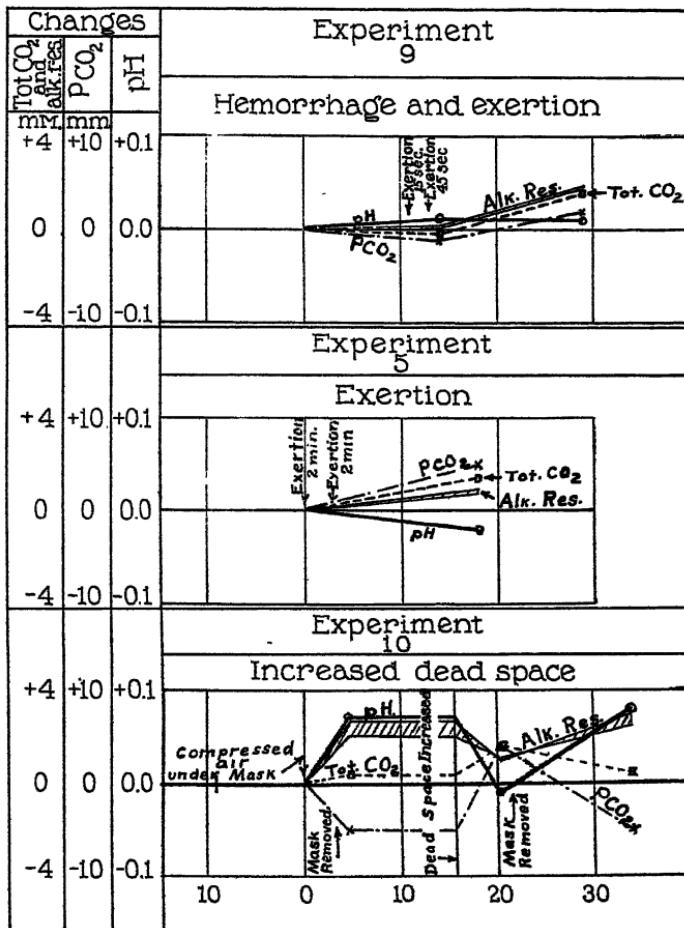


FIG. 3.

cated in the graphs. The changes in alkali reserve are all less than 5 mm. The changes observed in these experiments are not

entirely consistent with any given procedure. It is possible to interpret the group in either of two ways. The first is: that exertion of the degree present in these experiments is without effect on the alkali reserve; that repeated bleedings tend to induce a very slight increase in the alkali reserve; that application of a mask over the dog's muzzle is different in its effect depending upon

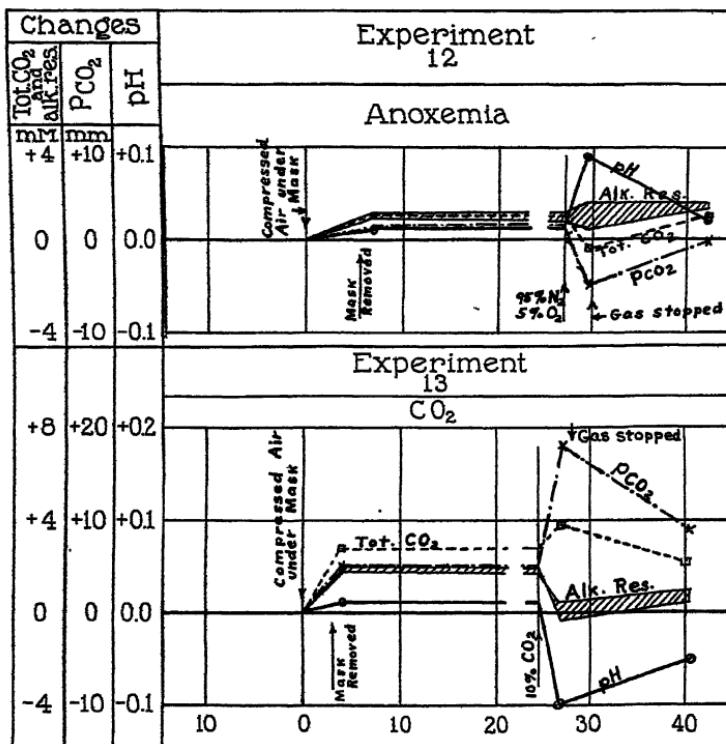


FIG. 4.

the efficiency of the ventilation under it, a slight rise in alkali reserve being observed with vigorous ventilation by streaming air through the mask, and a slight fall when increased dead space, diminished oxygen tension, or increased CO<sub>2</sub> tension are induced. The experiments with oxygen are, however, not entirely consistent with the interpretation given above. The second interpretation is based upon the fact that if the variations in alkali

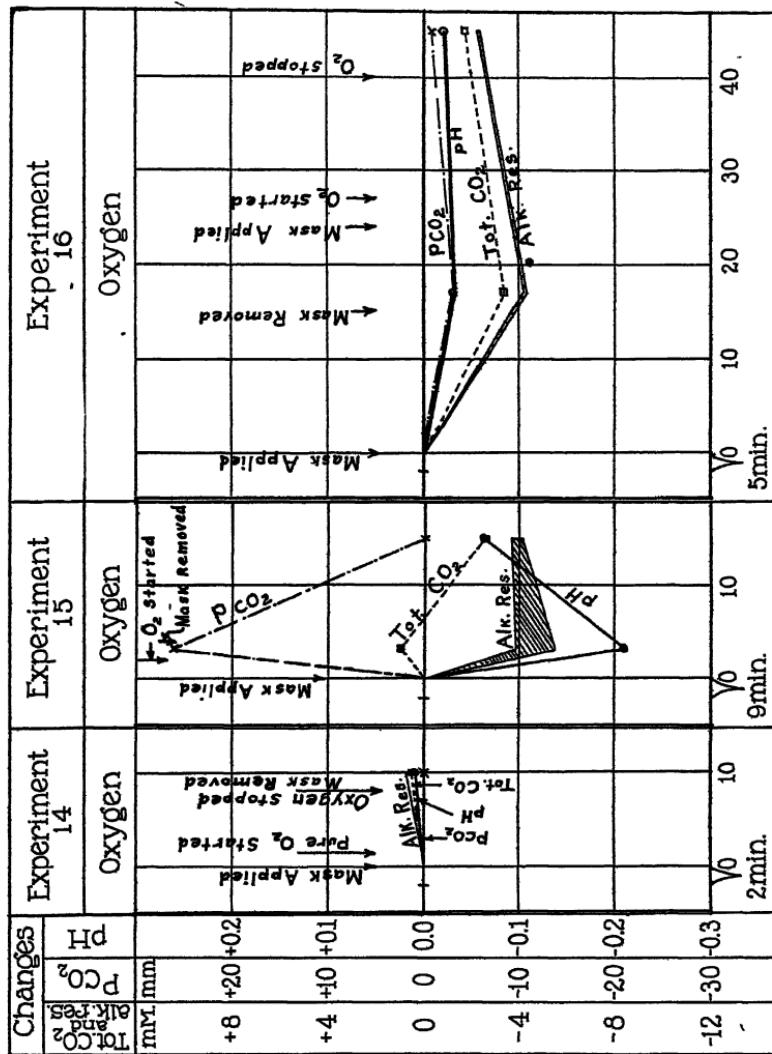


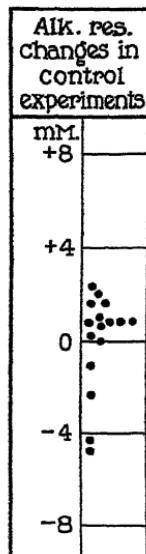
Fig. 5.

reserve in these control experiments are plotted regardless of the particular procedure adopted (see Fig. 6) it will be found that the changes distribute themselves in such a way that they might be interpreted as the fortuitous variations to be expected at repeated bleedings. Whichever interpretation be preferred, we may conclude that the control experiments indicate that the factors taken into consideration do not produce at least individually a change in alkali reserve greater than 5 mm.

### Group III.

(Experiments 6, 7, and 8; Fig. 7.)

*Nitrous Oxide Anesthesia.*—The effect of nitrous oxide when administered either pure or as 95 per cent nitrous oxide and 5 per cent oxygen is shown in Experiments 6 and 7.



In both these experiments it was found impossible to secure anesthesia in the dog without using 95 per cent or more of  $\text{N}_2\text{O}$ . This inevitably led to marked anoxemia. There occurred at first a rise in total  $[\text{CO}_2]$  in the serum associated in Experiment 6 with a marked increase in pH and a very low calculated  $\text{CO}_2$  tension. An attempt to correct for the oxygen unsaturation and to extrapolate the  $[\text{BHCO}_3]$  to the initial pH gives results indicative of a rise in alkali reserve at the time of the second bleedings. Whether this was true or whether certain of the assumptions made in our calculations were invalidated by the suddenly and extremely altered conditions in the blood at this time we are not prepared to say. At the third bleeding, however, 5 minutes after beginning the administration of the gas there had

occurred a fall in the alkali reserve to 4.4 mm. or more below the initial level. This fall in alkali reserve was associated in Experiment 6 with a marked fall in the total  $[\text{CO}_2]$  of the blood with pH still above its initial level; in Experiment 7 at the third bleeding with a similar fall in alkali reserve the total  $[\text{CO}_2]$  was the same as at the initial observation, but the pH had fallen 0.08. These differences in total  $[\text{CO}_2]$  and

in pH, in spite of similar falls in the level of the  $\text{CO}_2$  absorption curve as measured by the fall in alkali reserve must be attributed to differences in pulmonary ventilation. The position of the blood along the  $\text{CO}_2$  absorption curves at the third bleeding is quite different in the two experiments, the  $\text{CO}_2$  tension being much lower in Experiment 6.

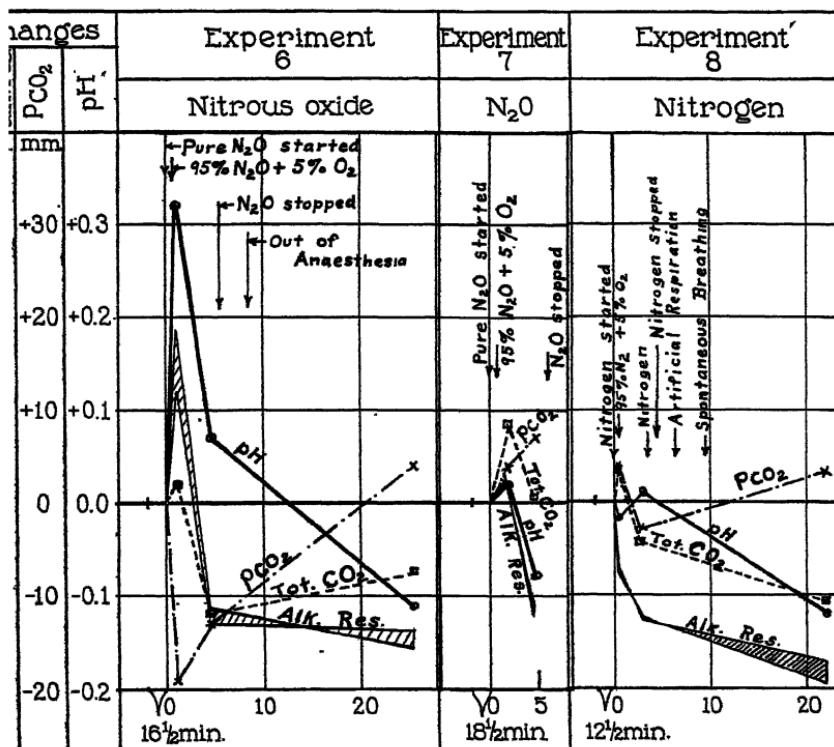


FIG. 7.

*Anoxemia.*—In order to determine how much of this effect reported above might be attributed to the anoxemia alone, Experiment 8 was performed in which nitrogen was administered for 30 seconds followed by a mixture of 95 per cent nitrogen and 5 per cent  $\text{O}_2$ . The results corresponded closely with those obtained in Experiment 7, except that there was no initial rise in the calculated alkali reserve. In Experiment 12 in which there

was administered 95 per cent nitrogen and 5 per cent oxygen, but at no time pure nitrogen, and in which the unsaturation of the blood probably did not exceed 3.5 mm. the fall in alkali reserve was slight if indeed it occurred at all. In the presence of marked anoxemia, therefore, this fall in alkali reserve may occur; on the other hand, a very considerable degree of anoxemia may occur without fall in alkali reserve. The fall associated with nitrous oxide is no greater than that occurring with an equal degree of anoxemia produced by a nitrogen-oxygen mixture. It is interesting to compare the initial acidosis in these experiments with the slowly developing acidosis due to anoxemia reported by Loevenhart and his colleagues.

The fall in alkali reserve in these experiments is as rapid as in the ether and chloroform experiments, but it is less marked. Indeed in magnitude it lies just at the border of the change observed in our group of control experiments. It is possible that the change represents the effect of the most extreme anoxemia that can be produced, compatible with continued life, or it may be due to factors identical with those operating in the chloroform and ether experiments. Our decision must await further studies of the nature of the initial acidosis.

#### DISCUSSION.

In the experiments with ether and chloroform the persistently low pH, high CO<sub>2</sub> tension, and increased oxygen unsaturation of the blood suggest a depression of the sensitiveness of the respiratory center during anesthesia. Possibly the final observation of Experiments 6 and 8 following extreme anoxemia are to be similarly interpreted.

From a consideration of the entire series of experiments it will be seen that rise or fall of the alkali reserve of a few mm., even of as much as 5 mm., may occur associated apparently with slight disturbances of the respiratory mechanism. A positive explanation of these smaller changes in alkali reserve is not afforded by our experiments.

There is a consistent, rapid, and more marked, fall in alkali reserve amounting to from 4.5 to 8 mm. which constantly occurs in the first few minutes of ether or chloroform anesthesia.

A less marked but equally rapid fall in alkali reserve is caused by inhalation of either  $N_2O$  or  $N_2$  containing not more than 5 per cent of oxygen.

Control experiments upon exertion as great as that occurring in the anesthetic experiments but without interference with respiration, showed no fall in alkali reserve. The discrepancy between these results and those found by Barr, Himwich, and Green in exercise is probably due to the difference in the amount of exertion.

It is possible that exertion during anoxemia is an important factor in this fall. These two factors, exertion and anoxemia, in varying degrees have been present in all of the experiments showing marked fall of alkali reserve, and all experiments where both of these factors were present have exhibited some fall in the alkali reserve. These factors, however, do not seem to be the entire explanation of the fall observed with ether and chloroform.

The rapidity with which the fall in alkali reserve occurs upon administering anesthetics together with the fact that further fall becomes less and less pronounced as the anesthesia proceeds and becomes deeper, suggests that this initial fall may be not a specific effect of the anesthetic or of anesthesia but a more general phenomenon of disturbed respiration, circulation, or tissue activity. The fact that in the ether and chloroform experiments the fall in pH precedes the fall in total  $[CO_2]$  indicates that the fall in alkali reserve is primary and not a withdrawal of base to compensate for excessive loss of  $CO_2$  from hyperventilation.

#### SUMMARY.

1. The larger part of the fall in alkali reserve that occurs with ether, chloroform, and nitrous oxide anesthesia occurs during the first few minutes of the anesthesia.
2. The fall in alkali reserve with nitrous oxide anesthesia is less than with ether and chloroform. A fall of the same degree and rate of development can be produced by using nitrogen instead of nitrous oxide in the same concentration.
3. Exertion, psychic disturbance, and repeated bleedings as occurring in these experiments do not of themselves produce a fall in alkali reserve.

4. Anoxemia alone without exertion lowers the alkali reserve, but less than does the administration of the anesthetics.

5. The importance of the combination of anoxemia and exertion is suggested by these experiments. These are probably not the only factors responsible for the fall in alkali reserve in these experiments.

6. The immediate fall in pH associated with the fall in alkali reserve indicates that we are dealing with an uncompensated acidosis and not with a compensated alkalosis.

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*Experiment 1.**Experiment to Test Immediate Effect of Ether Anesthesia.*

Young male, No. 6, weight 10 kilos. 9.44 a.m. First venous bleeding from right jugular vein, 70 cc. 9.52 a.m. First cardiac bleeding from left ventricle, 70 cc.; dog struggled moderately. 10.01 a.m. Ether started; struggle vigorous but brief; ether by drop method, first with towel, then gauze. 10.02 to 10.03  $\frac{1}{2}$  a.m. Second cardiac bleeding from left ventricle, 70 cc. 10.16 to 10.17  $\frac{1}{2}$  a.m. Second venous bleeding from right jugular, 70 cc. 10.19 to 10.20 a.m. Third, cardiac bleeding from left ventricle, 70 cc. 10.20 a.m. Ether stopped.

Cardiac sample No.	Time drawn.	True serum as drawn.					
		Determined.		Calculated.			
		Total $[CO_2]$ , mM.	Colorimetric pH at 38°.	$CO_2$ tension as drawn, mm.	$[BHCO_3]$ , mM.	$\frac{d[BHCO_3]}{dpH}$ , mM.	$[BHCO_3]$ at initial pH, mM.
1	Before ether.....	24.9	7.43	35	23.8	23.8	23.8
2	After 2 min. ether.....	24.1	7.17	60	22.2	17.0	14.9
3	" 18 " " .....	18.8	7.25	39	17.6	14.0	12.6

*Experiment 2.**Showing Effect of Chloroform Anesthesia.*

Young male, No. 6, weight 10 kilos. 9.40 a.m. First bleeding from left heart, 15 cc.; quiet. 9.45 to 9.46 a.m. Put under chloroform suddenly. Moderate struggling for 40 seconds. 9.46 a.m. Second bleeding from left heart, 15 cc.; blood darker. 9.50 a.m. Almost out of anesthesia; third bleeding from left heart, 15 cc.; blood brighter. 9.51 a.m. Breathing violently while taking latter half of blood. 9.59 a.m. Almost stopped breathing. 10.00 a.m. Respirations returning. 10.01 a.m. Breathing regular and fast; fourth bleeding from left heart, 15 cc.

Sample No.	Time drawn.	True serum as drawn.					
		Determined.		Calculated.		[BHCO <sub>3</sub> ] at initial pH.	[BHCO <sub>3</sub> ] = — 20 dpH
		Total [CO <sub>2</sub> ] mM.	Colorimetric pH at 38°.	[BHCO <sub>3</sub> ] mM.	[CO <sub>2</sub> tension as drawn. mm.]		
1	Before chloroform.....	23.57	7.27	47	22.08	22.1	22.1
2	After 1 min. chloroform..	22.89	7.11	64	20.86	17.7	16.4
4	" 16 " "	17.97	7.12	49	16.40	13.4	12.2

*Experiment 3.**Showing Immediate Effect of Chloroform Anesthesia.*

Young male, No. 6, weight 10 kilos. 10.59 a.m. On table. 11.18 a.m. Bled from right ventricle, 15 cc. 12.03 p.m. First bleeding from left ventricle, 15 cc. 12.10 p.m. Rectal temperature 38.4°, pulse 82, respirations 24. 12.16 p.m. Chloroform administration begun by drop method on gauze. 12.17 p.m. Vigorous struggle. 12.18 p.m. Relaxing. 12.19 p.m. Completely relaxed. 12.21 p.m. Second bleeding, left ventricle, 15 cc. 12.22 p.m. Chloroform administration stopped.

Sample No.	Time drawn.	True serum as drawn.					
		Determined.		Calculated.			
		Total [CO <sub>2</sub> ] <i>m.M.</i>	Colorimetric pH at 38°.	[CO <sub>2</sub> tension as drawn. <i>mm.</i>	[BHCO <sub>3</sub> ] <i>m.M.</i>	[BHCO <sub>3</sub> ] <i>m.M.</i>	[BHCO <sub>3</sub> ] <i>m.M.</i>
1	Before chloroform.....	23.60	7.34	40	22.32	22.3	22.3
2	After 5 min. chloroform..	23.20	7.12	64*	21.18	16.8	15.0

\* This high CO<sub>2</sub> tension suggests that the pulmonary ventilation was inadequate at this time and that there was probably an increased oxygen unsaturation of the left ventricular blood. As no oxygen analyses are available in this experiment no correction has been made for this unsaturation; such a correction would have increased still further the fall in alkali reserve at the second bleeding.

*Experiment 4.**Showing Effect of Brief Chloroform Anesthesia.*

Young male, No. 6, weight 10 kilos. 10.38 a.m. Unsuccessful puncture. 10.43 a.m. Successful puncture, first bleeding, right ventricle, 15 cc. 10.45 a.m. Mask of gauze applied. 10.46½ a.m. Chloroform started, violent struggling for 1 minute. 10.48 to 10.49 a.m. Second bleeding, left ventricle, 15 cc. 10.51 a.m. Violent struggle for 45 seconds. 10.52 to 10.53 a.m. Third bleeding as soon as relaxed, left ventricle, 15 cc. 10.53 to 10.56 a.m. Fully under chloroform. 10.57 a.m. Chloroform pushed. 10.57 to 10.58 a.m. Fourth bleeding, left ventricle, 15 cc. 10.58 a.m. Stopped breathing just as blood tube was filled, artificial respiration. 11.05 a.m. Spontaneous breathing resumed.

Sample No.	Time drawn.	True serum as drawn.					
		Determined.		Calculated.			
		Total [CO <sub>2</sub> ] m.M.	Colorimetric pH at 38°. pH	[BHCO <sub>3</sub> ] m.M.	[BHCO <sub>3</sub> ] m.M.	$\frac{d[BHCO_3]}{dpH} = -20$	$\frac{d[BHCO_3]}{dpH} = -28$
1	Before chloroform.....	26.24	7.37	42	24.90*	23.3†	23.3†
2	After 1½ min. chloroform.	21.80	7.29	42	20.48	18.9	18.3
3	" 5½ " "	23.30	7.15	60	21.39	17.0	15.2
4	" 10½ " "	22.82	7.18	55	21.07	17.3	15.8

\* Not corrected for oxygen unsaturation.

† Corrected for oxygen unsaturation.

Blood 1 was drawn from the right ventricle, but as no oxygen determinations were made, accurate correction for O<sub>2</sub> unsaturation could not be made. The maximum unsaturation we have observed under these conditions is however, 3.17 mm. We have assumed this figure in calculating the [BHCO<sub>3</sub>] at initial pH and saturated.

*Experiment 5.*

*To Control Effect of Muscular Activity Such as Occurred in These Experiments.*

*To Show Early Effects of Chloroform Anesthesia.*

Young male, No. 5, weight 10 kilos. 10.03 a.m. First bleeding, 15 cc. 10.09 to 10.11 a.m. Vigorous exertion. 10.12 to 10.14 a.m. Vigorous exertion. 10.17 to 10.20 a.m. Muscles tense; animal restless throughout interval between bleedings even when not vigorously active. 10.27 a.m. Second bleeding, 15 cc. 10.38 a.m. Chloroform anesthesia commenced by drop method on gauze and continued until 10.53 a.m. 10.52 a.m. Third bleeding, 15 cc.

Sample No.	Time drawn.	True serum as drawn.						
		Determined.		Calculated.				
		Total $[CO_2]$ .	Colorimetric pH at 38°.	$[HCO_3^-]$	$\frac{d[HCO_3^-]}{dpH}$	$[HCO_3^-]$ at initial pH.	$\frac{d[HCO_3^-]}{dpH} = -20$	$[HCO_3^-]$ at 38°
1	Before exertion.....	22.25	7.36	36	21.09	21.1	21.1	
2	After " .....	23.75	7.34	41	22.46	22.1	22.0	
3	" 14 min. chloroform.	17.90	7.26	36	16.74	14.7	13.9	

*Experiment 6.**Showing Effect of 95 Per Cent N<sub>2</sub>O with 5 Per Cent O<sub>2</sub>.*

Young male, No. 6, weight 10 kilos. 9.53 a.m. First bleeding, 30 cc. 10.09½ a.m. Pure N<sub>2</sub>O administered 30 seconds. 10.10 a.m. Mixture of 95 per cent N<sub>2</sub>O and 5 per cent O<sub>2</sub> administered 5 minutes to 10.15 a.m. 10.10½ to 10.11 a.m. Second bleeding, 30 cc. 10.14 to 10.15 a.m. Third bleeding, 30 cc. 10.18 a.m. Out of anesthesia; quiet. 10.35 a.m. Fourth bleeding, 30 cc.

Sample No.	Time drawn.	True serum as drawn.						Whole oxalated blood as drawn.	
		Determined.		Calculated.					
		Total [CO <sub>2</sub> ].	Colorimetric pH at 38°.	CO <sub>2</sub> tension as drawn.	Total [CO <sub>2</sub> ].	[BHCO <sub>3</sub> ]	$\frac{d[BHCO_3]}{dpH} = -20$		
		m.M.	pH	mm.	m.M.	m.M.	$\frac{d[BHCO_3]}{dpH} = -25$		
1	Before gas.....	22.56	7.34	39	22.54	21.31	21.3	21.3	7.40.04
2	After 1 min. gas.....	23.40	7.66	20	20.20	19.66	26.1	28.7	6.76.15
3	" 5 " "	17.68	7.41	26	15.27	14.56	16.0	16.6	7.04.64
4	" 20 " recovery	19.60	7.23	43	19.39	18.05	15.9	15.0	6.50.40

*Experiment 7.**Showing Effect of 95 Per Cent N<sub>2</sub>O with 5 Per Cent O<sub>2</sub>.*

Young male, No. 6, weight 10 kilos. 9.53 a.m. First bleeding, 33 cc 10.11½ to 10.12 a.m. Pure N<sub>2</sub>O administered by mask. 10.12 to 10.17½ a.m. Mixture of 95 per cent N<sub>2</sub>O and 5 per cent O<sub>2</sub> administered by mask. 10.18½ to 10.14 a.m. Second bleeding, 33 cc. 10.16 to 10.17½ a.m. Third bleeding, 33 cc.

Sample No.	Time drawn.	True serum as drawn.										[O <sub>2</sub> ] unsaturation of whole oxalated blood as drawn.	
		Determined.		Calculated.									
		Total [CO <sub>2</sub> ].	Colorimetric pH at 38°.	CO <sub>2</sub> tension as drawn.		Total [CO <sub>2</sub> ].		[BHCO <sub>3</sub> ]	d[BHCO <sub>3</sub> ]/dpH = -20	[BHCO <sub>3</sub> ]	d[BHCO <sub>3</sub> ]/dpH = -28		
mM.	pH	mm.	mm.	mM.	mM.	mM.	mM.	mM.	mM.	mM.	mM.	mM.	
1	Before gas.....	22.70	7.36	37	22.40	21.23	21.2	21.2	21.2	21.2	21.2	0.52	
2	After 2 min. gas.....	26.04	7.38	41	22.97	21.83	22.2	22.2	22.2	22.4	22.4	5.91	
3	" 5 " "	22.72	7.28	44	19.65	18.43	16.8	16.8	16.2	16.2	16.2	5.91	

*Experiment 8.**Showing Effect of Mask with 95 Per Cent Nitrogen and 5 Per Cent Oxygen.*

Young male, No. 6, weight 10 kilos. 10.04 a.m. First bleeding, 37 cc. 10.16½ a.m. Pure nitrogen 30 seconds followed by 95 per cent N<sub>2</sub> and 5 per cent O<sub>2</sub> until 10.20 a.m., then pure nitrogen 60 seconds; mask removed at 10.21 a.m. 10.17 to 10.18 a.m. Second bleeding, 37 cc. 10.19½ to 10.20½ a.m. Third bleeding, 34 cc. 10.23 a.m. Spontaneous respiration ceased and manual artificial respiration was employed for 3 minutes. 10.26 a.m. Breathing spontaneously, conscious, quiet. 10.38½ to 10.39½ a.m. Fourth bleeding, 37 cc.

Sample No.	Time drawn.	True serum as drawn.						Whole oxalated blood as drawn.			
		Determined.		Calculated.							
				Corrected for O <sub>2</sub> unsaturation.							
		Total [CO <sub>2</sub> ].	Colorimetric pH at 38°.	CO <sub>2</sub> tension as drawn.	Total [CO <sub>2</sub> ].	[BHCO <sub>a</sub> ]	d[BHCO <sub>a</sub> ] = -20	d[BHCO <sub>a</sub> ] = -28	[O <sub>2</sub> ] capacity.	[O <sub>2</sub> ] unsaturation.	
		m.M.	pH	mm.	m.M.	m.M.	m.M.	m.M.	m.M.	m.M.	
1	Before anoxemia.....	24.16	7.40	36	23.9	22.8	22.8	22.8	(8.6)	0.5	
2	After 1 min. anoxemia.	25.60	7.38	40	21.5	20.4	20.0	19.8	8.6	7.9	
3	" 3½ " "	22.45	7.41	33	18.5	17.5	17.7	17.8	(8.6)	7.7	
4	Reoxygenated.....	20.00	7.28	39	19.6	18.4	16.0	15.0	8.1	0.8	

*Experiment 9.**To Control Effect of Repeated Bleedings and of Such Muscular Activity as Occurred in These Experiments.*

Young male, No. 6, weight 10 kilos. 9.42 a.m. First bleeding, 37 cc. 9.53 a.m. Vigorous exertion for 45 seconds. 9.56 a.m. Second bleeding, 37 cc. 10.11 a.m. Third bleeding, 37 cc.

Sample No.	True serum as drawn.					Whole oxalated blood as drawn.		
	Determined.		Calculated.			[O <sub>2</sub> ] capacity.	[O <sub>2</sub> ] unsaturation.	
	Total [CO <sub>2</sub> ]. m.M.	pH	Colorimetric pH at 38°. mm.	[BHCO <sub>3</sub> ] m.M.	$\frac{d[BHCO_3]}{dpH}$ at -20	$\frac{d[BHCO_3]}{dpH}$ at -28		
1	23.00	7.34	39	21.75	21.8	21.8	7.9	0.04
2	22.82	7.35	38	21.61	21.8	21.9	8.0	0.04
3	24.65	7.35	41	23.34	23.5	23.6	7.7	0.02

*Experiment 10.**Showing Effect of Well Ventilated Mask and Later of the Mask with Dead Space Increased by 200 Cc.*

Young male, No. 7, weight 20 kilos. 9.42 to 9.43 a.m. First bleeding from left ventricle, 36 cc. 9.51 $\frac{1}{2}$  to 9.55 a.m. Compressed air passed through mask. 9.56 $\frac{1}{2}$  to 9.57 $\frac{1}{2}$  a.m. Second bleeding from left ventricle, 36 cc. 10.07 to 10.12 $\frac{1}{2}$  a.m. Mask applied, dead space increased by means of rubber tubing to the extent of 200 cc., respirations deeper, but no change in respiratory rate. 10.11 $\frac{1}{2}$  to 10.12 $\frac{1}{2}$  a.m. Third bleeding from left ventricle, 36 cc. 10.25 $\frac{1}{2}$  to 10.26 $\frac{1}{2}$  a.m. Fourth bleeding from left ventricle, 36 cc.

Sample No.	Time drawn.	True serum as drawn.						Whole oxalated blood as drawn.	
		Determined.		Calculated.					
		Total [CO <sub>2</sub> ].	Colorimetric pH at 38°.	[CO <sub>2</sub> tension as drawn.]	Total [CO <sub>2</sub> ].	[BHCO <sub>3</sub> ]	[BHCO <sub>3</sub> ] at initial pH.		
m.M.	pH	mm.	mm.	m.M.	m.M.	m.M.	d[BHCO <sub>3</sub> ] = dpH	d[BHCO <sub>3</sub> ] = dpH	
1	Before mask was applied.....	23.71	7.37	38	23.20	22.02	22.0	22.0	8.20 0.98
2	After mask was applied.....	24.14	7.44	33	23.77	22.73	24.1	24.7	8.00 0.71
3	4 $\frac{1}{2}$ min. after dead space was increased.....	25.23	7.36	42	24.56	23.28	23.1	23.0	7.91 1.29
4	12 $\frac{1}{2}$ min. after mask was removed.....	24.20	7.45	33	23.85	22.83	24.4	25.0	7.70 0.67

*Experiment 12.*

*Showing Effect of Application of Well Ventilated Mask and Later of an Atmosphere of 95 Per Cent N<sub>2</sub> and 5 Per Cent O<sub>2</sub>.*

Young male, No. 7, weight 20 kilos. 9.57 to 9.58½ a.m. First bleeding from left ventricle, 36 cc. 10.08 to 10.13½ a.m. Compressed air under mask; quiet. 10.15 to 10.16 a.m. Second bleeding from left ventricle, 36 cc. 10.35 to 10.38 a.m. Nitrogen 95 per cent, oxygen 5 per cent under mask. Slower and deeper breathing. 10.37½ to 10.38½ a.m. Third bleeding from left ventricle, 36 cc.; quite restless. 10.50½ to 10.51½ a.m. Fourth bleeding from left ventricle, 36 cc.; quiet.

Sample No.	Time drawn.	True serum as drawn.						Whole oxalated blood as drawn.	
		Determined.		Calculated.					
		Total [CO <sub>2</sub> ] mM.	Colorimetric pH at 38°. pH	[CO <sub>2</sub> tension as drawn. mm.]	Total [CO <sub>2</sub> ]. mM.	[BHCO <sub>3</sub> ] mM.	$\frac{d[BHCO_3]}{dpH} = -20$ mM.	$\frac{d[BHCO_3]}{dpH} = -28$ mM.	
1	Before mask was applied.....	20.49	7.38	32	19.95	18.96	19.0	19.0	9.1 1.03
2	After mask was applied.....	21.40	7.39	33	20.86	19.84	20.0	20.1	8.6 1.03
3	2½ min. after nitrogen started.....	20.00	7.47	27	18.8	18.03	19.8	20.6	8.4 2.23
4	12½ min. after nitrogen stopped .....	21.41	7.40	32	21.01	20.01	20.4	20.6	8.0 0.76 3.5*

\* In this experiment the oxalated blood was drawn just after the blood from which the true serum was taken. The nitrogen administration was stopped and the mask removed between the filling of the two tubes. On the basis of the color of the blood, the unsaturation of the blood from which the true serum was taken was probably nearer 3.5 mm. than 2.23 mm.

*Experiment 13.**Showing Effect of Application of Well Ventilated Mask and Later of an Atmosphere of Air Containing 10 Per Cent CO<sub>2</sub>.*

Young male, No. 12, weight 21 kilos. 9.46 to 9.47 a.m. First bleeding from left ventricle, 36 cc. 9.57 to 9.60 a.m. Compressed air under mask, quiet. 10.01 to 10.01½ a.m. Second bleeding from right ventricle, 36 cc. 10.21½ to 10.25 a.m. 10 per cent CO<sub>2</sub> under mask. 10.23½ to 10.25½ a.m. Third bleeding from left ventricle, 36 cc. 10.37½ to 10.38½ a.m. Fourth bleeding from left ventricle, 36 cc.

Sample No.	Time drawn.	True serum as drawn.						Whole oxalated blood as drawn.	
		Determined.		Calculated.					
		Total [CO <sub>2</sub> ] m.M.	Colorimetric pH at 38°. pH	CO <sub>2</sub> tension as drawn. mm.	Total [O <sub>2</sub> ]. m.M.	[BHCO <sub>3</sub> ] m.M.	[BHCO <sub>3</sub> ] dpH = - 20 m.M.	[BHCO <sub>3</sub> ] dpH = - 28 m.M.	
1	Before mask was applied.....	19.94	7.30	37	19.62	18.45	18.5	18.5	9.1 0.58
2	4 min. after mask was applied.....	22.82	7.31	42	21.29	20.06	20.3	20.4	8.7 2.94
3	10 per cent CO <sub>2</sub> under mask.....	23.75	7.20	55	22.57	20.92	18.9	18.1	8.2 2.28
4	12½ min. after CO <sub>2</sub> was stopped.....	22.06	7.25	46	21.81	20.37	19.4	19.0	7.8 0.49

*Experiment 14.*  
*Showing Effect of Mask with Oxygen.*

Male, No. 7, weight 20 kilos. 9.30 a.m. First bleeding, 68 cc. 9.32 a.m. Mask applied. 9.33 $\frac{1}{2}$  to 9.40 a.m. Pure oxygen administered through mask. 9.40 a.m. Mask removed. 9.42 a.m. Second bleeding, 68 cc.

Sample No.	Time drawn.	True serum as drawn.								[O <sub>2</sub> ] unsaturation of whole oxalated blood as drawn. m.M.	
		Deter-		Calculated.							
		Total [CO <sub>2</sub> ] m.M.	Colorimetric pH at 38°. pH	CO <sub>2</sub> tension as drawn. mm.	[BHCO <sub>1</sub> ] m.M.	$\frac{d[BHCO_1]}{dpH} = -20$	[BHCO <sub>2</sub> ] m.M.	$\frac{d[BHCO_2]}{dpH} = -28$			
1	Before O <sub>2</sub> .....	24.2	7.41	36	23.07	23.1	23.1	0.62			
2	After O <sub>2</sub> .....	24.7	7.42	36	23.57	23.8	23.9	0.54			

*Experiment 15.**Showing Effect of Mask with Oxygen.*

Young male, No. 6, weight 10 kilos. 10.25 a.m. First bleeding, 37 cc. 10.34 a.m. Mask applied, vigorous exertion 30 seconds. 10.36 to 10.38 a.m. Pure oxygen introduced through mask. 10.37 $\frac{1}{2}$  to 10.38 a.m. Second bleeding, 37 cc. 10.38 a.m. Mask removed. 10.49 a.m. Third bleeding, 37 cc.

Sample No.	Time drawn.	True serum as drawn.						Whole oxalated blood as drawn.	
		Deter- mined.		Calculated.					
		Total [CO <sub>2</sub> ].	Colorimetric pH at 38°.	CO <sub>2</sub> tension as drawn.		Corrected for O <sub>2</sub> unsaturation.			
		m.M.	pH	mm.	Total [CO <sub>2</sub> ].	[BEHCO <sub>3</sub> ]	$\frac{d[BHCO_3]}{dpH} = -20$	$\frac{d[BHCO_3]}{dpH} = -28$	
1	Before mask.....	23.2	7.31	43	22.7	21.4	21.4	21.4	
2	After 2 min. O <sub>2</sub> .....	24.2	7.10	69	24.0	21.8	17.6	15.9	
3	11 min. after O <sub>2</sub> stopped	20.7	7.25	43	20.2	18.9	17.7	17.2	
							[O <sub>2</sub> ] capacity.	[O <sub>2</sub> ] unsaturation.	

*Experiment 16.**Showing Effect of Mask over Muzzle and Subsequent Effect of Oxygen.*

Young male, No. 11, weight 12 kilos. 9.29 a.m. First bleeding, 36 cc. 9.34 a.m. Mask applied for study of ventilation rate and kept in place; dog absolutely quiet until 9.49 a.m. 9.51 a.m. Second bleeding, 36 cc. 9.58 a.m. Mask applied and ventilation rate measured. 10.01 to 10.14 a.m. Pure oxygen introduced through mask, ventilation rate being recorded. 10.19 a.m. Third bleeding, 36 cc.

Sample No.	Time drawn.	Ventilation rate. <i>per min.</i>	True serum as drawn.				Whole oxalated blood as drawn.	
			Determined.		Calculated.			
			Total [CO <sub>2</sub> ]. <i>mM.</i>	Colorimetric pH at 38°. <i>pH</i>	[BHCO <sub>3</sub> ] <i>mM.</i>	Corrected for O <sub>2</sub> unsaturation. [BHCO <sub>3</sub> ] at initial pH. $\frac{d[BHCO_3]}{dpH} = -20$		
1	Before mask.....	1.5—2.6	22.92	7.39	35	21.72	21.7	8.93 0.18
2	After mask.....	2.6—2.8	19.55	7.36	32	18.13	17.5	8.90 0.80
3	" " with oxygen.....		21.23	7.37	34	19.88	19.5	9.74 0.54

*Experiment 17.**Experiment to Follow Recovery of Alkali Reserve after Cessation of Ether Anesthesia.*

Young male, No. 7, weight 19 kilos. 9.38 a.m. First bleeding from left ventricle, 36 cc. 9.40 a.m. Ether started, struggle brief; ether by drop method with towel. 9.42 a.m. Animal under ether; etherization continued by streaming air over ether and into mask over animal's muzzle; anesthesia very light, conjunctival reflex never lost, occasional stiffening of the legs. 9.52 a.m. Ether insufficient to maintain anesthesia, vigorous struggle, etherization continued by drop method on two layers of gauze over muzzle. 9.54 a.m. Vomited, light anesthesia by drop method continued. 10.03 a.m. Second bleeding, left ventricle, 36 cc. 10.04 a.m. Etherization stopped. 10.08 a.m. Conscious, panting. 10.12 a.m. Off table, restless. 10.32 a.m. Third bleeding probably from right ventricle, 18 cc. 10.42 a.m. Drank small amount of water. 11.03 a.m. Fourth bleeding, left ventricle, 18 cc. 11.35 a.m. Fifth bleeding, right ventricle, 18 cc., discarded. 12.33 p.m. Sixth bleeding, left ventricle, 18 cc. 2.32 p.m. Seventh bleeding, right ventricle, 18 cc.

Sample No.	Time drawn.	True serum as drawn.						Whole oxalated blood as drawn.	
		Deter- mined.		Calculated.					
		Total [CO <sub>2</sub> ] m.M.	Colorimetric pH at 38°.	Corrected for O <sub>2</sub> unsaturation.		[BHCO <sub>2</sub> ] <sub>st</sub> initial pH. m.M.	[BHCO <sub>2</sub> ] <sub>-20</sub> dpH m.M.	[BHCO <sub>2</sub> ] <sub>-28</sub> dpH m.M.	
				Total [CO <sub>2</sub> ] m.M.	[BHCO <sub>2</sub> ] m.M.				
1	Before ether.....	26.5	7.42	38	26.1	24.9	24.9	24.9	9.7 0.7
2	After 23 min. ether....	21.3	7.23	46	19.4	18.1	14.3	12.8	10.8 3.7
3*	" 28 " recovery.	19.5	7.19	46	18.0	16.6	12.0	10.2	(3.0)†
4	" 59 "	19.5	7.21	44	19.1	17.8	13.6	11.9	(0.7)†
6	" 2½ hrs. "	24.1	7.32	43	23.7	22.4	20.4	19.6	(0.7)†
7*	" 4½ "	24.5	7.28	48	22.9	21.5	18.7	17.6	(3.0)†

\* Blood from right ventricle.

† Approximate values, no analyses.

## Experiment 18.

*To Determine the Effect of Brief Nitrous Oxide Anesthesia upon the Electrometric pK<sub>i</sub> of True Serum.*

Young male, No. 7, weight 19 kilos. 9:30 a.m. On table. 9:34 to 9:39 a.m. First bleeding, 98 cc. 9:46 a.m. Mask applied. 9:49 a.m. Pure N<sub>2</sub>O administered. 9:50 a.m. Under gas, but lid reflex present; gas changed to 95 per cent N<sub>2</sub>O with 5 per cent O<sub>2</sub>. 9:51 a.m. Slight rigidity and struggle. 9:52 a.m. Mask removed. 9:52 to 9:53 a.m. Struggle, then quiet to end of experiment. 9:59 to 10:03 a.m. Second bleeding, 98 cc.

Sample No.	True serum as drawn.		True serum equilibrated at 38°.		Whole oxalated blood as drawn.	
	Determined.	Calculated.	Determined.	Calculated.	[O <sub>2</sub> ] unsaturation.	[O <sub>2</sub> ] capacity.
Time drawn.						
1	Before gas.....	23.187	23.42	33	21.79	21.8
2	8 min. after recovery from gas .....	22.327	27.37	36	20.15	19.2

Time drawn.	Total [CO <sub>2</sub> ].		Colorimetric pH at 38°.		Total [CO <sub>2</sub> ].		Electrometric pH at 38°.		[HCO <sub>3</sub> ] <sub>i</sub> .		Colorimetric pH at 20°.		pK <sub>i</sub> (electrometer).		[O <sub>2</sub> ] capacity.		Whole oxalated blood as drawn.	
	m.M.	pH	m.M.	m.M.	m.M.	m.M.	m.M.	m.M.	m.M.	m.M.	m.M.	m.M.	m.M.	m.M.	m.M.	m.M.	m.M.	
1	23.187	42	33	21.79	21.8	21.8	39.2	23.48	7.33	33	21.8	21.8	39.2	23.48	7.33	1.25	22.23	6.08
2	22.327	27.37	36	20.15	19.2	18.8	39.8	22.32	7.31	7.31	17.65	16.06	21.06	6.09	10.12	0.09	7.07	

Corrected for O<sub>2</sub> unsaturation.

[BHCO<sub>3</sub>]<sub>i</sub> at initial pH.

d[pH] = -20

[BHCO<sub>3</sub>]<sub>i</sub> = -28

d[pH] = -28

[BHCO<sub>3</sub>]<sub>i</sub> = -38

d[pH] = -38

[BHCO<sub>3</sub>]<sub>i</sub> = -48

d[pH] = -48

[BHCO<sub>3</sub>]<sub>i</sub> = -58

d[pH] = -58

[BHCO<sub>3</sub>]<sub>i</sub> = -68

d[pH] = -68

[BHCO<sub>3</sub>]<sub>i</sub> = -78

d[pH] = -78

[BHCO<sub>3</sub>]<sub>i</sub> = -88

d[pH] = -88

[BHCO<sub>3</sub>]<sub>i</sub> = -98

d[pH] = -98

[BHCO<sub>3</sub>]<sub>i</sub> = -108

d[pH] = -108

[BHCO<sub>3</sub>]<sub>i</sub> = -118

d[pH] = -118

[BHCO<sub>3</sub>]<sub>i</sub> = -128

d[pH] = -128

[BHCO<sub>3</sub>]<sub>i</sub> = -138

d[pH] = -138

[BHCO<sub>3</sub>]<sub>i</sub> = -148

d[pH] = -148

[BHCO<sub>3</sub>]<sub>i</sub> = -158

d[pH] = -158

[BHCO<sub>3</sub>]<sub>i</sub> = -168

d[pH] = -168

*Experiment 19.*

To Compare the Change in Colorimetric and Electrometric pH of the True Serum after Injection of HCl.  
 Young male, No. 9, weight 13 kilos, 9.40 a.m. First bleeding, 118 cc. 10.04 to 10.10 a.m. Injected 23 cc. N HCl into left ventricle. 10.47 a.m. Second bleeding.

Sample No.	Determined.	True serum as drawn.	Calculated.	Whole oxalated blood as drawn.
		A Colorimetric pH.	Corrected for O <sub>2</sub> unsaturation.	[O <sub>2</sub> ] unsaturation.
		Total [CO <sub>2</sub> ].	[BHCO <sub>3</sub> ] $\frac{d\text{pH}}{d[\text{BHCO}_3]} = -20$	[O <sub>2</sub> ] capacity.
		CO <sub>2</sub> tension as drawn.	[BHCO <sub>3</sub> ] $\frac{d\text{pH}}{d[\text{BHCO}_3]} = -28$	[O <sub>2</sub> ] m.M.
		A Colorimetric pH at 38°.	[BHCO <sub>3</sub> ] $\frac{[\text{BHCO}_3] \text{ at initial pH.}}{[\text{BHCO}_3] \text{ at } 38^\circ} = -28$	m.M.
	Time drawn.	Electrometric pH at 38°.		m.M.
1	Before acid.....	pH m.M.	pH m.M.	23.6
2	After acid.....	7.62 26.20	7.17 7.31	24.90 48
		7.22	-0.14	23.60
			19	23.60
			7.43	9.4
			6.91	2.41
			4.1	8.6
			3.0	0.36

*Experiment 20.**To Compare Change in Colorimetric and Electrolytic pH Following Brief Ether Anesthesia.*

Young male, No. 7, weight 20 kilos. 9:35 a.m. First bleeding, 118 cc. 9:45 a.m. Etherized with ether soaked towel and light even anesthesia continued by drop method on gauze until 10:08 a.m. 10:15 a.m. Animal conscious. 10:40 a.m. Second bleeding, 135 cc.

Example No.	Time drawn.	Determined.		Calculated.		True serum as drawn.		Whole oxalated blood as drawn.		[O <sub>2</sub> ] unsaturation.	
		Total [CO <sub>2</sub> ] <sup>a</sup> .	[CO <sub>2</sub> tension as drawn.]	Total [CO <sub>2</sub> ] <sup>a</sup> .	[CO <sub>2</sub> tension as drawn.]	[BHCO <sub>3</sub> ] <sup>b</sup>	[BHCO <sub>3</sub> ] <sup>b</sup> at init. 1 pH.	[BHCO <sub>3</sub> ] <sup>b</sup> = -28	[BHCO <sub>3</sub> ] <sup>b</sup> = -28	[O <sub>2</sub> ] espacity.	[O <sub>2</sub> ] m.M.
1 Before ether.....	22.98	pH 7.42	pH 7.40	pH 7.42	pH 7.40	m.M. 33	m.M. 22.56	m.M. 21.48	m.M. 21.5	m.M. 10.0	m.M. 0.80
2 After ether.....	19.85	pH 7.28	pH 7.29	pH 7.28	pH 7.29	-0.14	-0.11	39	18.25	17.14	14.3



## A STUDY OF THE CALCIUM BALANCE OF DAIRY COWS.\*

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### *Résumé of Previous Work.*

The literature on the subject of calcium metabolism is large and only such pieces of work as appear to have some bearing on the work reported here are reviewed. It was found by Hart, McCollum, and Humphrey that low phosphorus intake was accompanied by an increased output of calcium in the urine, apparently involving a calcium-phosphorus entity in the body.

Negative balances of calcium, magnesium, and phosphorus were obtained by Forbes, Beegle, Fritz, Morgan, and Rhue, in a metabolism trial with dairy cows. Increasing the intake of these minerals by the use of clover and alfalfa hays, calcium carbonate, and bone flour, decreased the losses, but did not make the balances positive. Further investigations on this problem by Forbes, Halverson, Morgan, Schulz, Mangels, Rhue, and Burke resulted in negative balances. By using alfalfa hay as the sole roughage and supplementing the feed with bone flour, calcium lactate, and calcium chloride, a large intake of the mineral elements was secured.

Patterson found that during calcium starvation the ratio of calcium in the blood to the total ash in the blood remained much the same as in the normal animal. This work points to the bones as the seat of calcium loss and also of calcium storage. The concentration of calcium in the blood plasma of cows was shown by Meigs, Blatherwick, and Cary (1919, *a*) to be quite constant, although small variations could be induced by varying the amount supplied in the ration. They suggest the concentration of bicarbonate in the plasma as probably the chief controlling factor.

It is reported by Clark (1920) that the feeding of a calcium-rich diet had no effect on the calcium content of rabbit blood, although intravenous or subcutaneous injections of calcium salts may cause a transitory increase in the calcium content. On a calcium-rich diet Clark (1920-21) found that repeated subcutaneous injections of citrate, malate, or phosphate of cal-

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cium had no effect on the calcium balance of rabbits. On a low calcium intake, phosphate injection may cause a decrease of 20 to 26 per cent in the calcium content of the blood. It was found by Denis and Minot that the administration of calcium salts *per os* to men, cats, and rabbits did not increase the concentration of calcium in the plasma unless the initial concentration was low. Evidence is brought forward by Malcolm to show that the ingestion of soluble magnesium salts causes an increased loss of calcium in adult animals and hinders its deposition in young growing animals. Soluble calcium salts did not seem to effect the excretion of magnesium.

Soluble magnesium compounds introduced parenterally into animals were found by Mendel and Benedict (1909-10, *a*) to be excreted largely by way of the kidneys, the intestinal path being of minor significance. Increased output of urinary calcium followed the increased excretion of magnesium. The same authors (1909-10, *b*) also report the elimination by way of the kidneys of the excess of calcium chloride introduced intravenously. This increased excretion of calcium was accompanied by a rise in the urinary output of magnesium. Working with swine, Hart and Steenbock found that magnesium salts added to the ration increased the calcium elimination in the urine, but not in the fecal output. Soluble phosphates decreased the calcium excretion.

The results obtained by Givens (1917, *a*) afford evidence that diets poor in calcium are not conducive to a positive calcium balance. The relation of calcium to magnesium in the urine was almost always 1:1, but in the feces calcium always exceeded magnesium 2:1. On diets of mixed natural foods containing more magnesium than calcium, Givens (1918, *a*) found the daily calcium and magnesium urinary excretion of nine healthy adults ranged from 0.05 to 0.24 gm. of calcium and from 0.03 to 0.15 gm. of magnesium. When the diet contained more calcium than magnesium the limits were 0.12 to 0.47 gm. of calcium and 0.05 to 0.23 gm. of magnesium. In general more calcium than magnesium was excreted in the urine. In the case of twenty-five apparently healthy individuals, Nelson and Burns found that seventeen excreted larger amounts of calcium than magnesium in the urine, and in eight cases magnesium predominated over the calcium excretion. Whichever element predominated appeared to do so nearly constantly, and seemed to be independent of the character of the food.

It was found by Meigs, Blatherwick, and Cary (1919, *b*) that the assimilation of phosphorus and probably that of calcium in dry cows was favored by a system which reduced the disturbing influence of calcium in the intestine. This result was secured by feeding a double allowance of a phosphorus-rich grain ration 1 day without roughage, and feeding a double ration of a calcium-rich roughage the following day without grain. Increased milk yields were found by Meigs and Woodward to result when cows were fed, during the preceding dry period, a ration supplemented with disodium phosphate. The disodium phosphate was fed with the grain mixture and the grain and hay fed on alternate days.

The excretion of bases from the body, following the feeding of acid to a dog, was studied by Stehle. He found that ammonia was the greatest neutralizing factor, but that magnesium and calcium excretion in the urine also rose during the acid ingestion. By averaging the fecal output of these two elements during the acid period and likewise for the acid-free period, he found the output to be greater in the former. Consequently, he was led to conclude that the administration of hydrochloric acid increased the excretion of calcium and magnesium from the body. It was found by Givens and Mendel that the administration of base or acid produced no significant effect on the balance of magnesium or calcium. The ingestion of hydrochloric acid, however, increased the urinary calcium, thereby altering the relation of calcium to magnesium in the urine and presumably diverting lime from the intestinal path.

As a result of other experiments on dogs, Givens (1918, *b*) was able to confirm a previous statement that feeding hydrochloric acid was without marked influence on calcium and magnesium metabolism. Increased urinary elimination of calcium was again observed following the ingestion of hydrochloric acid or sodium chloride, but the absolute amount of this increase did not noticeably effect the calcium balance. The results of a metabolism trial on a growing pig by Lamb and Evvard show that the presence of lactic, acetic, and sulfuric acids in the ration did not cause any significant loss of calcium.

Poor utilization of fats and fatty acids, when the intake of calcium was comparatively abundant, was found by Givens (1917, *b*) to increase the lime in the feces. The negative balance was smaller in the cases where the utilization of fat was more complete. The large amount of calcium and phosphorus present in cow's milk was found by Bosworth, Bowditch, and Giblin to interfere with the fat absorption and utilization in infants suffering from constipation and disturbed fat metabolism. Ordinary cow's milk caused a great increase of insoluble calcium soaps in the feces. Decalcified milk brought about increased fat digestion and absorption. It was found by Holt, Courtney, and Fales (1918) that there was not an abnormal amount of fat excreted as soap by infants receiving dilutions of cow's milk.

Extensive work on normal, rachitic, and diarrheal children led Holt Courtney, and Fales (1920, *a*) to conclude that the excretion and absorption was, in general, dependent on the amount of calcium intake. An excessive intake did not proportionally increase the calcium absorption, while very low intake resulted in very low absorption. The excretion of calcium as soap was never a large proportion of the calcium intake. The excretion of calcium was found by these authors (1920, *b*) to be not at all related to the fat intake. The calcium lost as soap was in most cases an insignificant part of the calcium intake. It is shown by Hutchison that there is no increase in the excretion of soaps in the feces of rachitic children and that the excessive loss of calcium in this connection is not brought about through the agency of fat in the diet.

It was noted by Steenbock and Hart that a ration of green grass supplied to a goat that had been confined for 3 months to dry feed, resulted in greatly improved physical health and a regular retention of lime. Studying the difference in the composition of milk on a dry fodder ration and a ration containing pasture grass, Hess, Unger, and Supplee found the amounts of calcium and phosphorus significantly higher in the pasture milk.

It is suggested by Hart, Steenbock, and Hoppert that there is present in green plant tissue an unknown factor influencing calcium assimilation. Green oats, oat hay dried in diffuse sunlight, and cod liver oil were all effective in reducing calcium excretion. Having conducted palatability tests of mineral supplements with cows belonging to a group which had received only dry feed and silage for a long period, Forbes commends the view of Hart, Steenbock, and Hoppert.

#### EXPERIMENTAL WORK.

Our mode of attack has been the alternation of two rations, one high in calcium and low in fat and the other low in calcium and high in fat. Our theory was that the loss in calcium might be due to the formation of insoluble calcium soaps excreted in the feces, and that by keeping the calcium and fat apart such loss might be avoided.<sup>1</sup>

In the two trials all factors were kept as nearly uniform as possible, though different amounts were used and the feeds were varied to some extent. In each case three cows were used and the trial lasted for 90 days. Each trial period was divided into three periods of 30 days. In each period the ration was kept uniform and allowance made for all refused feed. The first 20 days of each period were used as a transition period and only the last 10 days were actually used in the work and reported here.

In each of the three experimental periods of 10 days, an accurate record of feed consumption by each cow was kept. They were not allowed out for exercise during the experimental periods, but had exercise in a dry lot daily during the preliminary periods. The cows were watered morning and evening and were weighed before and after drinking. The morning weights after feeding hay and before watering were used to determine the average

<sup>1</sup> Credit is due Dr. A. W. Dox, formerly Chief of the Chemistry Section, Iowa Agricultural Experiment Station, who suggested this procedure. The authors are also indebted to Dr. S. B. Kuzirian, L. Yoder, Edith Wilson, J. A. Schulz, J. Waddell, and other members of the staff who assisted in some of the determinations and other work in connection with this project.

live weights of the animals. Salt rolls were kept in front of them at all times and were dried and weighed at the end of each 10 day period. All roughages and grains were fed twice daily. The cows were milked twice daily and the milk was weighed.

In each experimental period the urine and feces were collected, by the usual methods, and stored in tared vessels until the end of each 24 hour period when they were weighed and sampled. Samples of feces, urine, and milk were taken daily for both immediate analysis and storage, while in each experimental period a representative sample of each feed was prepared. Organic and inorganic analyses were made of all the samples by the recognized methods. From the data, balances for a number of organic and inorganic constituents were obtained, but it was felt that only those which might apparently be connected with calcium balance should be reported here.

TABLE I.  
*Animals Used in Trial I.*

Cow No.....	305	308	309
Breed.....	Holstein.	Grade Guernsey.	Ayrshire.
Age, yrs.—mos.—days.....	2-6-16	2-6-6	2-5-27
Fresh, days.....	72	93	103
Bred, days.....			
Previous lactations.....			

### *Trial I.*

The animals used in this work were three young cows in milk and not bred. Table I contains information concerning them, and where necessary, it is calculated to the 1st day of the experiment; namely, February 17, 1918.

The experiment consisted of three experimental periods of 10 days, each preceded by a preliminary period of 20 days. The feeds used during this work were arranged to present the following characteristics:

Period No.	General character of ration.
I	High in fat, low in calcium, no free acid.
II	Low in fat, high in calcium, no free acid.
III	High in fat, low in calcium, free acid.

The amount of feed consumed by each animal during each period is given in Table II, while the yields of milk, feces, and urine are given in Table III. To provide succulence in Period I, one-half

TABLE II.  
*Feed Consumed in Trial I.*

Period No.	Feed.	Cow No.		
		305 lbs.	308 lbs.	309 lbs.
I	Cracked corn.....	30	20	25
	Gluten feed.....	30	20	25
	Wheat bran.....	33	20	27
	Oil meal (old process).....	17	10	13
	Timothy hay.....	140	100	130
II	Ground oats.....	27	25	30
	Wheat bran.....	26	25	30
	Alfalfa hay.....	120	90	120
	Beet pulp.....	30	30	30
III	Cracked corn.....	20	17	20
	Ground oats.....	20	17	20
	Oil meal (old process).....	20	16	20
	Timothy hay.....	60	50	50
	Corn silage.....	250	150	250

TABLE III.  
*Yields of Milk, Feces, and Urine and Average Live Weights in Trial I.*

Period No.	Cow No.	Milk. lbs.	Feces. lbs.	Urine. lbs.	Average live weights. lbs.
I	305	226.9	549.9	149.6	1,072
	308	108.3	333.4	73.6	833
	309	171.9	430.1	93.5	987
II	305	169.7	326.0	182.3	989
	308	103.3	224.9	105.3	804
	309	164.0	300.5	121.5	935
III	305	183.3	597.7	100.2	1,007
	308	108.1	398.1	80.5	836
	309	192.5	481.5	99.5	968

of the hay was chopped and mixed with the oil meal and bran, and fed as a mash in the morning. Soaked beet pulp provided the succulence in the second period and corn silage in the third.

#### DISCUSSION OF RESULTS.

Data regarding the income and outgo balances for the three periods are presented in Tables IV, V, and VI. The outstanding features of the data contained in these tables are the negative mineral balances of Periods I and III and the positive balances of Period II.

An inspection of the fat intake shows that there was very little difference in the amounts ingested in the three periods. Cows 308 and 309 actually received more fat in their ration in Period II than in Period I. It is apparent, therefore, that the amount of fat present in the feed cannot be held wholly responsible for the great differences in calcium retention that existed in the different periods.

The production of insoluble calcium soaps in the feces is presumably the only way in which fat could cause the loss of calcium from the body. The excretion of soaps in the feces did not vary greatly in any of the periods, and from the data presented, it appears that it might be an individual characteristic. Thus in all three periods, Cow 305 excreted the greatest amount of soap in the feces, followed by Cow 309, and with Cow 308 excreting the least. The intake of fat by Cow 305 in Period I was 6.30 lbs., coupled with a calcium oxide intake of 0.54 lb. The figures for Cow 309 were 5.31 lbs. of fat and 0.47 lb. of calcium oxide, while those for Cow 308 were 4.11 lbs. of fat and 0.36 lb. of calcium oxide. Hence it might be supposed from the data of Period I alone that the excretion of soap was in proportion to the intake of fat and calcium. However, in Period II, Cow 309 ingested more fat and more calcium than did Cow 305 and in Period III they received the same ration, while, as stated above, Cow 305 always excreted more soap than did Cow 309. Again, on examining the data for Cows 308 and 309 it is seen that in Periods I and III they received less fat and much less calcium than they did in Period II, and yet they excreted more soap in their feces in the two periods of low intake. It would therefore seem that the amount of fat fed in the rations during this investigation had no consistent effect on the excretion of soaps in the feces.

During Period II, when the ration was high in calcium, the amount of this element excreted in the feces was greatly increased over that of the other periods. It was during this period, however, that the three animals excreted the least amount of soap in the feces. It would appear, therefore, that the production of calcium soaps is not a significant channel for the excretion of an excess of calcium.

TABLE IV.  
*Income and Outgo Balances of Trial I, Period I (10 Days).*

Cow No.		Total fat.	Fatty acid fat.	Neutral fat.	Fat as soap.	Total ash.	P <sub>2</sub> O <sub>5</sub>	CaO	MgO	N
		lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.
305	Milk.....	7.38	0.03	7.35		1.57	0.45	0.39	0.02	1.00
	Urine.....					2.44	0.01	0.01	0.06	1.66
	Feces.....	2.38	0.76	1.62	0.32	9.02	1.43	0.38	0.82	1.76
	Total outgo..	9.76	0.79	8.97	0.32	13.03	1.89	0.78	0.90	4.42
	Total income	6.30	3.46	2.84	1.03	7.74	2.17	0.54	0.83	4.19
	Balance.....	-3.46	2.67	-6.13	0.71	-5.29	0.28	-0.24	-0.07	-0.23
308	Milk.....	5.72	0.02	5.70		0.78	0.28	0.22	0.03	0.58
	Urine.....					1.80	0.01	0.01	0.06	1.10
	Feces.....	1.60	0.47	1.12	0.10	6.70	1.20	0.33	0.50	1.17
	Total outgo..	7.32	0.49	6.82	0.10	9.28	1.49	0.56	0.59	2.85
	Total income	4.11	2.22	1.89	0.67	5.26	1.36	0.36	0.51	2.71
	Balance.....	-3.21	1.73	-4.93	0.57	-4.02	-0.13	-0.20	-0.08	-0.14
309	Milk.....	7.63	0.02	7.61		1.24	0.40	0.29	0.03	0.89
	Urine.....					1.98	0.03	0.01	0.04	1.43
	Feces.....	1.88	0.56	1.32	0.16	9.12	1.51	0.39	0.69	1.46
	Total outgo..	9.51	0.58	8.93	0.16	12.34	1.94	0.69	0.76	3.78
	Total income	5.31	2.87	2.44	0.87	6.61	1.79	0.47	0.67	3.50
	Balance.....	-4.20	2.29	-6.49	0.71	-5.73	-0.15	-0.22	-0.09	-0.28

Silage was fed in Period III in order that some free organic acid might be introduced into the ration. No appreciable effect on the calcium content of the urine of the animals in this trial is apparent. The intake of calcium during this period, although slightly greater than that of Period I, is low and the balances of

calcium do not, as a whole, differ greatly from those of Period I. The urinary output of magnesium is increased in this period, however, over that of the other periods, while the fecal magnesium is less. This would indicate that the free organic acids of the ration may have caused a diversion of magnesium from the intes-

TABLE V.  
*Income and Outgo Balance of Trial I, Period II (10 Days).*

Cow No.		Total fat.		Neutral fat.	Fat as soap.	Total ash.	P <sub>2</sub> O <sub>5</sub>	CaO	MgO	N
		lbs.	Fatty acid fat.							
305	Milk.....	5.46	0.02	5.44		1.22	0.31	0.25	0.03	0.81
	Urine.....					3.96	0.01	0.04	0.07	1.97
	Feces.....	1.69	0.44	1.25	0.16	12.84	1.01	3.13	0.75	1.34
	Total outgo..	7.15	0.46	6.69	0.16	18.02	1.33	3.42	0.85	4.12
	Total income	5.80	2.67	3.13	1.39	19.33	1.58	4.92	1.01	4.83
	Balance.....	-1.35	2.21	-3.56	1.23	1.31	0.25	1.50	0.16	0.71
308	Milk.....	4.93	0.01	4.92		0.75	0.28	0.20	0.03	0.63
	Urine.....					2.72	0.01	0.03	0.08	1.28
	Feces.....	1.39	0.34	1.05	0.10	8.19	0.81	2.77	0.58	1.08
	Total outgo..	6.32	0.35	5.97	0.10	11.66	1.10	3.00	0.69	2.99
	Total income	4.87	2.31	2.55	1.16	14.56	1.39	3.78	0.87	3.95
	Balance.....	-1.45	1.96	-3.42	1.06	2.90	0.29	0.78	0.18	0.96
309	Milk.....	6.71	0.02	6.70		1.23	0.36	0.26	0.05	0.87
	Urine.....					3.88	0.01	0.01	0.09	1.76
	Feces.....	1.61	0.42	1.19	0.13	13.22	1.05	3.52	0.78	1.23
	Total outgo..	8.32	0.44	7.89	0.13	18.33	1.42	3.79	0.92	3.86
	Total income	6.07	2.86	3.21	1.45	18.01	1.70	4.93	1.05	4.98
	Balance.....	-2.25	2.42	-4.68	1.32	-0.32	0.28	1.14	0.13	1.12

tine to the kidneys, due perhaps to a more ready absorption of the magnesium salts of these acids. The balances of magnesium in this period are not largely affected, and are positive in two cases while the balances of Period I, the most comparable in the matter of magnesium intake to Period III, are all negative.

In Period II, high calcium intake was secured by feeding alfalfa hay and beet pulp. Where the average calcium intake for the

three cows in Periods I and III was 0.46 and 0.72 lb., respectively, the intake during this period averaged 4.54 lbs. This high intake is reflected in the increased excretion in the feces. The most significant feature of this period is the positive balances of the mineral elements, particularly the relatively high calcium

TABLE VI.  
*Income and Outgo Balance of Trial I, Period III (10 Days).*

Cow No.		Total fat.	Fatty acid fat.	Neutral fat.	Fat as soap.	Total ash.	P <sub>2</sub> O <sub>5</sub>	CaO	MgO	N
		lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.
305	Milk.....	5.55	0.01	5.53		1.30	0.37	0.27	0.04	0.86
	Urine.....					2.14	0.01	0.01	0.19	1.04
	Feces.....	1.97	0.75	0.63	0.23	9.86	0.84	0.66	0.54	1.73
	Total outgo..	7.52	0.76	6.16	0.23	13.30	1.22	0.94	0.77	3.63
	Total income	6.04	3.04	3.00	0.62	9.34	1.18	0.80	0.86	4.90
	Balance.....	-1.48	2.28	-3.16	0.39	-3.96	-0.04	-0.14	0.09	1.27
308	Milk.....	5.35	0.01	5.34		0.79	0.29	0.22	0.03	0.66
	Urine.....					1.93	0.01	0.02	0.17	0.83
	Feces.....	1.71	0.65	1.06	0.16	6.93	0.80	0.64	0.52	1.39
	Total outgo..	7.06	0.66	6.40	0.16	9.65	1.10	0.88	0.72	2.88
	Total income	4.44	2.36	2.09	0.51	7.37	0.91	0.55	0.60	3.45
	Balance.....	-2.62	1.70	-4.31	0.35	-2.28	-0.19	-0.33	-0.12	0.57
309	Milk.....	7.76	0.02	7.75		1.39	0.44	0.33	0.04	1.06
	Urine.....					2.18	0.01	0.01	0.18	0.90
	Feces.....	1.71	0.67	1.04	0.20	8.57	0.82	0.67	0.58	1.54
	Total outgo..	9.47	0.69	8.79	0.20	12.14	1.27	1.01	0.80	3.50
	Total income	6.04	3.04	3.00	0.62	8.97	1.18	0.80	0.86	4.90
	Balance.....	-3.43	2.35	-5.79	0.42	-3.17	-0.09	-0.21	0.06	1.40

retention as compared with the period immediately preceding it and that immediately following it. The balance data for this 10 day period show that calcium oxide was stored in amounts varying from 1.5 to 0.78 lbs. per cow.

From work done on the calcium metabolism of goats, Hart, Steenbock, and Hoppert were led to believe that green plant tissue contains an unknown factor which favorably influences

calcium assimilation. They also suggest that fresh alfalfa hay may contain some of this unknown factor since it was effective in increasing calcium retention. The results of this work would seem to indicate that either the high calcium content of

TABLE VII.  
*Animals Used in Trial II.*

Cow No.....	91	166	187
Breed.....	Ayrshire.	Jersey.	Guernsey.
Age, yrs.—mos.—days .....	12-2-16	7-11-10	9-7-19
Fresh, days.....		88	178
Bred, days.....			85
Previous lactations.....	6	4	6

TABLE VIII.  
*Feed Consumed in Trial II.*

Period No.	Feed.	Cow No.		
		91	166	187
I	Ground oats.....	20	50	50
	Wheat bran.....	20	50	50
	Alfalfa hay.....	60	60	60
	Roots.....	500	400	500
II	Cracked corn.....	13½	33½	33½
	Wheat bran.....	13½	33½	33½
	Oil meal.....	13½	33½	33½
	Oat hay.....	80	80	80
III	Cracked corn.....	13½	33½	33½
	Wheat bran.....	13½	33½	33½
	Oil meal.....	13½	33½	33½
	Oat hay.....	40	40	40
	Corn silage.....	300	250	300

the alfalfa, or the possibility of its possessing this unknown substance, or both factors were responsible for the favorable balances of calcium. In any instance, the practice of using alfalfa, or some such calcium-rich legume in the dairy ration would seem

TABLE IX.

*Yields of Milk, Feces, and Urine and Average Live Weights in Trial II.*

Period No.	Cow No.	Milk.	Feces.	Urine.	Average live weight.
		lbs.	lbs.	lbs.	lbs.
I	91		220.5	247.5	951
	166	146.4	275.0	243.0	990
	187	181.2	426.5	357.0	953
II	91		236.0	155.0	985
	166	119.1	340.0	155.0	974
	187	99.0	494.0	173.0	963
III	91		402.0	160.0	1,062
	166	125.3	445.0	113.0	1,030
	187	101.3	646.0	103.0	1,000

TABLE X.

*Ash Balance of Trial II, Periods I, II, and III.*

Period No.....	I			II			III		
	P <sub>2</sub> O <sub>5</sub>	CaO	MgO	P <sub>2</sub> O <sub>5</sub>	CaO	MgO	P <sub>2</sub> O <sub>5</sub>	CaO	MgO
Cow No.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.
91	Milk.....								
	Urine.....	0.11	0.02	0.04	0.10	0.01	0.02	0.06	0.01
	Feces.....	1.17	2.29	0.75	0.54	0.83	0.57	1.25	1.73
	Total outgo.	1.28	2.31	0.79	0.64	0.84	0.59	1.31	1.74
	Total income	1.23	1.57	0.83	1.06	0.46	0.50	1.33	0.90
	Balance.....	-0.05	-0.74	0.04	0.42	-0.38	-0.09	0.02	0.07
166	Milk.....	0.13	0.32	0.03	0.11	0.29	0.02	0.12	0.28
	Urine.....	0.01	0.00	0.10	0.01	0.00	0.01	0.01	0.02
	Feces.....	1.93	2.48	0.85	1.63	0.78	0.78	1.96	0.38
	Total outgo..	2.07	2.80	0.98	1.75	1.07	0.81	2.09	1.68
	Total income	2.26	1.67	1.19	2.10	0.60	0.92	2.30	0.95
	Balance.....	0.19	-0.13	0.21	0.35	-0.47	0.11	0.21	-0.73
187	Milk.....	0.17	0.42	0.04	0.10	0.20	0.02	0.10	0.26
	Urine.....	0.02	0.01	0.18	0.00	0.01	0.01	0.01	0.02
	Feces.....	2.26	2.52	1.07	2.17	0.79	0.84	2.13	1.55
	Total outgo..	2.45	2.95	1.29	2.27	1.00	0.87	2.24	1.83
	Total income	2.30	1.69	1.23	2.10	0.61	0.92	2.37	1.06
	Balance.....	-0.15	-1.26	-0.06	-0.17	-0.39	0.05	0.13	-0.77

to be amply supported. In addition, the results of this trial would also tend to indicate that the fat in the ration and the presence of free organic acid have no influence on calcium metabolism.

### *Trial II.*

In the second trial conditions were somewhat similar to those in Trial I though different animals were used and the feeds were not quite the same. The animals used are given in Table VII

TABLE XI.  
*Nitrogen Balance of Trial II.*

Cow No.		Period No.		
		I	II	III
91	Milk.....			
	Urine.....	2.15	1.04	1.41
	Feces.....	1.10	0.78	1.37
	Total outgo.....	3.25	1.82	2.78
	Total income.....	3.00	1.76	2.78
	Balance.....	-0.25	0.06	0.00
166	Milk.....	0.83	0.70	0.83
	Urine.....	1.80	2.23	1.60
	Feces.....	0.99	0.88	1.29
	Total outgo.....	3.62	3.81	3.72
	Total income.....	4.15	3.68	4.48
	Balance.....	0.53	-0.13	0.76
187	Milk.....	0.98	0.60	0.64
	Urine.....	3.21	2.89	1.69
	Feces.....	2.13	1.88	2.52
	Total outgo.....	6.32	5.37	4.85
	Total income.....	4.24	3.68	4.69
	Balance.....	-2.08	-1.69	-0.16

and, where necessary, the data are calculated to November 18, 1919, the day on which the trial started. Cow 91 was dry and barren throughout the trial.

The feeds consumed in each of the three experimental periods in Trial II are listed in Table VIII, while the yields of milk, feces, and urine and the average live weights of the animals for each period are given in Table IX.

The balance data are presented in the same form as in the previous trial, but only the lime, magnesia, and phosphoric acid have been considered (Table X). The nitrogen balances are given separately in Table XI.

#### DISCUSSION OF RESULTS.

In Trial II, Period I corresponds to Period II in Trial I, in that it is the period of high calcium intake, while Periods II and III of Trial II correspond to Periods I and III of Trial I. However, it is found that throughout Trial II negative balances of calcium are found in every case, while those for magnesium and phosphoric acid are very variable. It is especially noticeable in this case that during high calcium intake the greatest negative calcium balances were found. This may have been due to the lack of the hypothetical substance already mentioned as aiding calcium storage. The alfalfa hay used in the second trial was  $1\frac{1}{2}$  years of age, and the substance aiding in the calcium assimilation, if present when the hay was fresh, may have been destroyed during the storage.<sup>2</sup>

#### SUMMARY.

1. It has been demonstrated in two metabolism trials that the fat content of the ration and the loss of calcium as insoluble soaps in the feces, are not important factors in the calcium balance of dairy cows.

<sup>2</sup> Since the above was written, Hart and his associates have published (Hart, E. B., Steenbock, H., Hoppert, C. A., and Humphrey, G. C., *J. Biol. Chem.*, 1922, liii, 21) evidence secured with liberally milking cows showing that it was possible to maintain a positive calcium balance with dry alfalfa hay, corn silage, and a grain mixture. The hay was of excellent quality, having been cured under caps. On the other hand the use of an alfalfa hay cured in the windrow with exposure to air and light for 4 days failed to bring about calcium equilibrium in a later experiment as reported in another paper (Hart, E. B., Steenbock, H., Hoppert, C. A., Bethke, R. M., and Humphrey, G. C., *J. Biol. Chem.*, 1922, liv, 75) in which the authors state: "These differences in effect of the two alfalfa hays may be attributed to a difference in the degree of destruction during the curing process of the vitamine assisting calcium assimilation." And, in more recently published metabolism experiments on women, Bogert and Trail (Bogert, L. J., and Trail, R. K., *J. Biol. Chem.*, 1922, liv, 387) obtained results which "suggest some influence of the vitamine content of the diet upon calcium assimilation."

2. In the trials here reported the acids of corn silage seemed to have no influence on the calcium balance.

3. A positive calcium balance was obtained in one period where good alfalfa hay of the previous season's crop was used, but when alfalfa hay 1½ years old was used, the calcium balance was negative even with a dry cow. This is in harmony with the theory of the presence of a vitamine in green leaves influencing calcium assimilation, which is destroyed with age or improper curing.

4. In every case where alfalfa hay was not fed a negative calcium balance was found.

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## DOES THE CHICK REQUIRE THE FAT-SOLUBLE VITAMINS?

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Sugiura and Benedict (1) in reporting some interesting results respecting the nutritional requirements of pigeons, state: ". . . fat-soluble vitamine is not essential in any stage of avian nutrition." They base this broad conclusion upon their work with squabs and pigeons—apparently drawing the somewhat natural inference that the vitamin requirements of all avian species are the same.

In the present paper, we wish to put forth data to substantiate in particular the assertion made by us (2): "Continuing the study of comparing the chick with the rat and the pigeon, the findings relative to the fat-soluble vitamins indicate, in the case of the White Leghorn breed, that it is fairly suitable for test purposes. In marked contrast with the pigeon, the fat-soluble vitamin requirements of the chick are very much greater."

Our studies related to: (a) comparison of the rate of growth of baby chicks in confinement, fed on synthetic rations that were complete and deficient in the vitamins A and B; (b) the physiological effects produced by feeding the vitamin deficient diets; (c) the results obtained by treating the pathological chicks with definite amounts of preparations, rich in the particular vitamin required; and (d) a comparison of the vitamin requirements of young chicks with those of young rats and pigeons (not squabs). No attempt was made to follow the problem through to the stage of hatching the eggs as Sugiura and Benedict did.

Several investigators have used the chicken in nutrition studies under laboratory conditions. In some cases, they have not met with success. The difficulties seem to pertain in the main to the question of the proper

adjustment of roughage (3), protein (4), mineral salts, and vitamins. Furthermore, it is well recognized in the poultry industry that sanitation and control of temperature are of prime importance, especially with baby chicks. It is also imperative that the eggs used in incubating come from stock that has never been infected with diarrhea.

In respect to the vitamins, Hart, Halpin, and Steenbock (5) in 1917 found that pullets fed a mixture of corn and wheat meal did better when it was supplemented with a salt mixture, protein, and 2 per cent butter fat, to furnish fat-soluble vitamin A. Later (6), in 1922, these authors reported that the presence of the fat-soluble vitamins such as occur in cod liver oil, are essential to the proper development of the baby chick. They found that absence of these vitamins in the diets was chiefly responsible for leg weakness or rickets. It was also reported that the requirements of the chicks for the fat-soluble vitamin is comparatively larger than that of the rat, during the early period of growth.

Voegtlind and Myers (7) reported in 1918, data which showed that the presence or absence of 5 per cent of butter fat in the ration of squabs had a decisive effect in determining whether the squabs would mature or not. Hughes (8) fed chickens over a period of 6 months and stated that those fed a low fat-soluble vitamin ration were more susceptible to infection. Later, he reported that on a low fat-soluble vitamin ration the eggs from these hens were in turn lower in this vitamin than those from the normal controls.

Wilkins and Dutcher (9), in feeding white rice to cockerels, observed three cases of an eye condition which was corrected by feeding 1 gm. of butter. These authors later (10) raised the question as to the value of the fat-soluble vitamin in reporting their observations on the weight of the testes of cockerels. They found that when fresh green alfalfa was added to the rice diet it corrected the atrophy of the testes. Whether this was due to either of the vitamins A or B, or both, which alfalfa contains, could not be answered.

Guerrero and Concepcion (11) observed, in feeding fowl white rice, that some of them developed xerophthalmia between the 7th and 79th days. They concluded that rice is not only lacking in vitamin B but also in the fat-soluble A. Plimner and Rosedale (12) studied the vitamin requirements of baby chicks. They supplied the vitamins A, B, and C. Their conclusion was that the vitamin B was more essential than vitamins A and C. In fact, according to their data, leg weakness appeared to be due to vitamin B deficiency. Further, the vitamin B requirements varied directly with the increased amount of carbohydrates and fat. Nelson, Lamb, and Heller (13) reported that the vitamin A requirements of the rabbit were greater than those of rats or pigs. They were not able to make any definite statement regarding the vitamin needs of chickens other than that they were "hampered considerably in working with younger chicks due to the development of leg-weakness, which appears to be a general symptom of malnutrition in chickens."

In the light of our study and that of Hart, Halpin, and Steenbock (5, 6), leg weakness is an indication of fat-soluble A vitamin insufficiency.

#### EXPERIMENTAL.

We used throughout the White Leghorn breed of chick. The series of tests on the chicks were carried out between September, 1921 and September, 1922. In the beginning, we incubated the eggs. Later, we decided more uniform results would be obtained if we purchased day old chicks from an expert who would guarantee us select birds. The chicks were placed in suitable quarters and fed in accord with the best methods of practical procedure for 2 to 3 weeks. They were then transported to the laboratory, marked, weighed, and divided into representative groups for the nutrition trials. The all metal cages, provided with removal bottoms, were 24 × 24 × 24 inches. The floor was covered with pine shavings which served also for part of the roughage. Charcoal, grits, oyster shell, and tap water were always present. The ration was fed *ad libitum*. No accurate record could be made of the food consumed. The chicks were weighed at least twice a week.

The control synthetic ration consisted of:

	per cent
Protein (meat residue).....	18.6
Peptone.....	3.6
Salt mixture.....	3.4
Starch.....	36.1
Lactose.....	24.6
Crude fiber (roughage).....	5.0
Lard.....	5.0
Vitamin B extract.....	2.5
" A " .....	1.2

The deficient synthetic rations were exactly the same as the complete ration excepting that one of the vitamin extracts was withdrawn as conditions demanded, and an equivalent amount of starch added in place of it. White rice was also used in several cases as the diet.

The salt mixture was the same as that recommended by McCollum, Simmonds, and Pitz (14) for rats, No. 185. The meat residue represented the portion of chopped lean beef left after

thorough extraction with hot water and alcohol. Osborne and Mendel (15) have shown that such a product is adequate for growth as a source of protein. However, we took the precaution to add a small amount of vitamin-free peptone. Besides, the roughage furnished by the shavings, 5 per cent of thoroughly exhausted alfalfa meal was used. To supply vitamins A and B special extracts of alfalfa and wheat germ, respectively, were employed. The vitamin C used was furnished by a potent tomato extract.

In preparing these rations, water was added to the mixed ingredients to make a thick mass. This was then passed through a coarse sieve and dried. By "granulating" the rations in this way, they were rendered comparable with the physical condition and coarseness of regular chick feeds.

#### DISCUSSION.

*Comparison of Baby Chicks on Synthetic Rations.*—In Charts 1 to 5, the variations in the rate of growth of baby chicks are shown graphically. It will be seen that, as far as the vitamins are concerned, the effects produced were as follows: the most evident and rapid cessation of growth was with the no vitamin group (Chart 5); after this the vitamin B deficiency chicks (Chart 2) were affected; followed by the group lacking the fat-soluble A vitamin (Chart 3). It is to be observed that practically normal growth was obtained with the control vitamins A and B ration (Chart 1), and that the addition or absence of vitamin C had little or no influence. That is to say, during the early stages of the growth of the chick, the vitamin C (Chart 4), contrary to the notion of most feeders, was not essential. Whether its presence is needed later, is not evident from these data.

*Physiological Effects Produced by the Deficient Rations.*—In Tables I to V are presented data showing the comparative effects resulting from feeding young chicks rations that were improperly balanced from the standpoint of vitamins. In Table I, the results indicate that these 84 chicks, when fed the control synthetic diet, manifested none of the symptoms associated with those diseases which are ascribed to vitamin deficiency in other small animals like the pigeon, rat, dog, or rabbit—such as beri-beri or polyneuritis, ophthalmia (xerophthalmia, xerosis, etc.), or leg weakness and rickets.

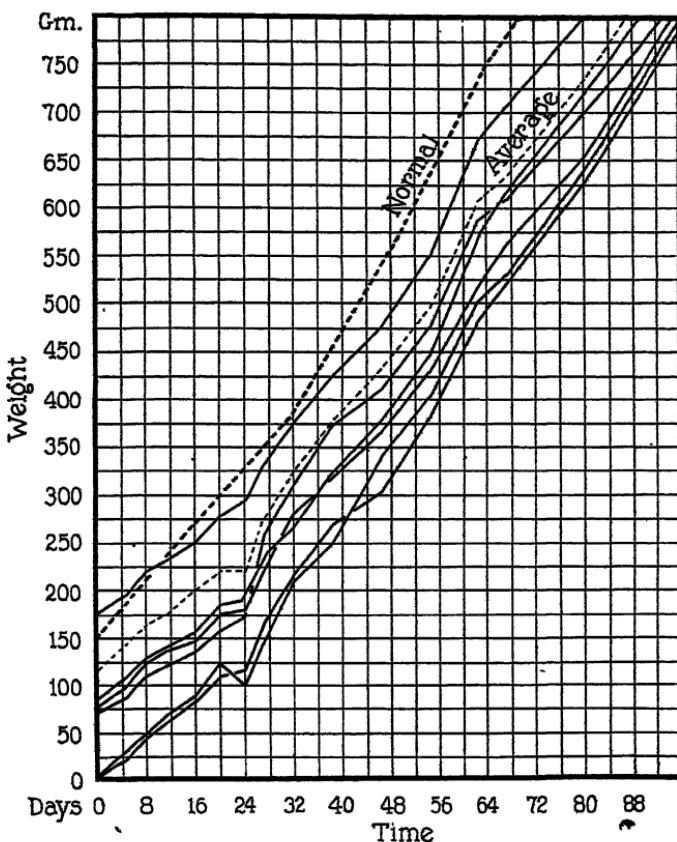


CHART 1. These chicks averaged 85 gm. on the 24th day when put on a synthetic ration. They grew at a normal rate, laid eggs on the 187th day, and were kept on the ration until they weighed 1,387 gm. Attention is called to the fact that no vitamin C was present.

*Complete Ration No. 7451.*

	per cent
Meat protein.....	18.6
Peptone.....	3.6
Salt mixture 185.....	3.4
Starch.....	36.1
Lactose.....	24.6
Lard.....	5.0
Roughage.....	5.0
Vitamin A extract.....	1.2
" B "	2.5

In the absence of vitamin B, Table II, 91 per cent of the 125 young chicks manifested beri-beri in varying degrees. This was evidenced by a cessation of growth, drooping of the wings, ruffled feathers, and general weakness. In many cases, the typical retraction of the head and paralysis were manifested. Death often occurred following the polyneuritic spasms in the case of baby chicks. In no instance did the animals on the vitamin B deficient diet show a typical case of ophthalmia or any rickets. The presence of urates was evidenced in 16 cases out of the 67 autopsied. Reference will again be made to this finding.

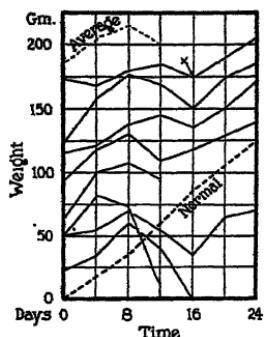


CHART 2.

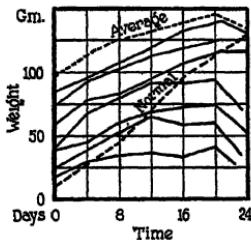


CHART 3.

CHART 2. The synthetic ration lacked vitamin B. At the point x all the surviving chicks were treated. Thereafter they gained and came back to normal.

CHART 3. Group 84. The synthetic ration lacked the fat-soluble vitamins. The chicks grew for a longer time before making the critical drop in weight, than did those on the vitamin B deficient diet.

When the fat-soluble vitamin A was withdrawn from the ration, it will be seen, Table III, that of the 185 cases reported 83 per cent were affected with an ophthalmic condition similar to that which occurs in rats, rabbits, and dogs when fed a like diet. The fact that the chicks were of different ages and hatchings, makes these findings more significant from the standpoint of undernutrition and infection. Further, it will be observed that the onset of the pathology became evident at varying times. For example, in Group AD 2, of the 37 chicks, the range in time varied from 14 to 62 days. There was no definite uniformity

as to when the condition occurred, except in the case of baby chicks. Here we found, in the instances where the tests began with chicks 10 to 14 days old that the effect of the deficiency was so pronounced that they died within a week or 10 days—often

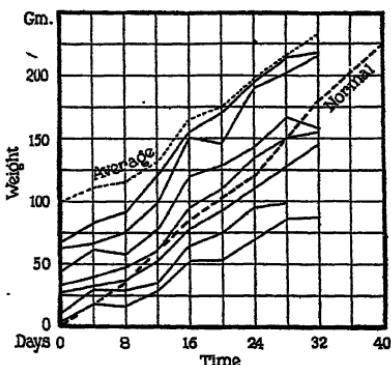


CHART 4.

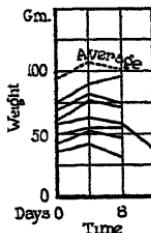


CHART 5.

CHART 4. In this experiment the chicks which were from the same hatch as those in Chart 1, were given an additional amount of a potent vitamin C extract which was more than sufficient to prevent scurvy in guinea pigs. This extra vitamin had no effect in stimulating the growth or activity of the chicks.

#### Ration 7451.

	per cent
Meat protein.....	18.6
Peptone.....	3.6
Salt mixture 185.....	3.4
Starch.....	36.1
Lactose.....	24.6
Lard.....	5.0
Roughage.....	5.0
Vitamin A extract.....	1.2
"    B    ".....	2.5
"    C    "  (separate treatment). .....	

CHART 5. Group 91. Ration contained no vitamins.

before the eye condition became very manifest. In other words, the occurrence of the ophthalmia was apparently influenced by the degree of lowered resistance that was brought about by the absence of the vitamin A.

## Vitamin Requirements of Chicks

In the case of the autopsies, it is of special interest to note the high percentage of incidences of urates. Beach (16) refers to a disease in poultry resembling roup and describes exactly what we have found with respect to the eyes, kidneys, and urates. He says, as to the urates: "They (kidneys) are usually very

TABLE I.  
*Complete Synthetic Diet (Control), Fed to Chicks.*

Group No.	No. of chicks.	Age. days	Initial weight. gm.	Ophthal-mia.	Rickets.	Beri-beri.	Urates.	Deaths.
CA 6	57	24	118	None.	None.	None.	None.	None.
CB 2	17	32	153	"	"	"	"	2
CC 1	6	39	358	"	"	"	"	None.
CD 1	4	46	293	"	"	"	"	"

TABLE II.  
*Vitamin B Deficient Diet, Fed to Chicks.*

Group No.	No. of chicks.	Age. days	Initial weight. gm.	Beri-beri.				Ophthal-mia.	Urates.		
				++	+	Total.	Percent-age. per cent		Autop-sies.	Total positive.	Cases. per cent
BA 6	58	24	112	15	37	52	90	None.*	45	11	24
BB 5	37	31	135	24	10	34	92	"	9	1	11
BC 1	19	34	150	11	8	19	100	"	13	4	30
BD 1	11	39	325	3	6	9	82	"	None.		
Total..	125			53	61	114	91	None.*	67	16	24

\* One chick had one bad eye. Local treatment did not correct the condition. 2 days after putting it on a balanced diet, the chick died; cause unknown.

pale and marked with a network of very fine white lines which are urate-filled tubules. We also frequently find a deposit of white material, probably urates, on the liver, heart and other organs."

The number of cases of rickets was relatively small in chicks that were started after they were 3 weeks old. Under this age, we had a high percentage with weak legs, even though they appeared thrifty at the time of beginning to feed the faulty vitamin ration.

TABLE III.  
*Vitamin A Deficient Diet, Fed to Chicks.*

Group No.	No. of chicks.	Ophthalmia.										Urates.				Rickets.
		Age. days	Initial weight. gm.	No.	Positives. per cent	No. of treated cases	No. of cured cases. per cent	Cured.	Day of occurrence.	Autopsies.	Positive cases.		Total.	Percentage. per cent		
											No.	No. of cured cases.	Slight.	Marked.		
AA 5	41	24	121	22	51	15	12	80	10-25	26	1	21	22	85		
AB 3	23	29	153	20	87	17	12	70	12-17	1		1	1	100		
AC 2	14	35	158	13	93	11	7	64	9-22	7		6	6	86		
AD 2	37	39	251	34	92	30	14	47	14-62	11	4	4	8	73	13	
AE 2	28	47	252	26	93	20	9	45	10-40	10	2	7	9	90		
AF 1	5		423	5	100	4	1	25	18-24	4	2	1	3	75		
AG 1	4	77	433	4	100	2	1	50	10	3	1	1	2	67		
AH 1	24		536	21	87	20	4	20	11-57	14	4	4	8	57	3	
AI 1	4		652	4	100	3	2	67	12-17	1		1	1	100	2	
AJ 1	5		767	4	80	4	1	25		2	1		1	50	2	
Total..	185			153	82.7	126	63	50	9-62	79	15	46	61	77	20	

TABLE IV.  
*Vitamins A and B Deficient Diet (Rice), Fed to Chicks.*

Group No.	No. of chicks.	Beriberi.					Ophthal- mia.	Rickets.		Treat- ments.		Urates.			Percentage. per cent
		Age. days	Initial weight. gm.	No.	Percentage. per cent	No.		No.	Per centage.	No.	Cures.	Autopsy.	Cases.		
RA 1	4	56	180	3	75	1	25			3	2	1	1	100	
RB 1	5	63	252	4	80	1	20			1		1	1	100	
RC 1	6	70	299	3	50	2	33			2	2	3	2	66	
RD 3	15		490	5	33	13	86	3	20	14	3	10	6	60	
RE 2	10		660	5	50	4	40	1	10	8	5	3	2	66	
Total....	40			20	50	21	52	4	10	28	12	18	12	66	

Again, it should be noted that none of the chicks on this diet had beri-beri or polyneuritis, but they showed symptoms which were specific and characteristic of the vitamin A deficiency. This point is brought out more clearly perhaps in Table IV where white milled rice was fed. This being deficient in both vitamins A and B, we should expect to have the occurrence of beri-beri (polyneuritis), ophthalmia, and the evidence of urates. Such was the case, the number of incidences varying with the particular condition, requirements, and idiosyncrasies of various animals. Thus, in Table II, on the vitamin B minus diet, it will be recalled that 24 per cent of the 67 chicks autopsied showed urates. The explanation for this would be that the food con-

TABLE V.  
*Summary of Influence of Deficient Vitamin Diets on Chicks.*

Diet deficient in.	Total No. of chicks.	Beriberi.		Ophthalmia.		Urates.	
		No.	Per- centage.	No.	Per- centage.	No.	Per- centage.
Vitamin A.....	185		per cent	153	83	79	77
" B.....	125	114	91	None.		16	24
" A and B.....	40	20	50	21	52	18	66
Complete.....	84	None.		None.		None.	

sumption of some of the chicks was so low that there was not quite enough vitamin A to prevent the disturbance which resulted in the accumulation of the urates, yet there was sufficient to retard the onset of the macroscopic appearances of ophthalmia.

*Alleviation of Ophthalmia with Vitamin A.*—In Table III, 126 cases of ophthalmia were treated orally and of these 63, or 50 per cent were cured. In many of those that did not respond the disease was too far advanced, while in others the eyes would probably have cleared up if we had given the proper dosage for the particular stage of the disease. The fact remains, however, that by simply furnishing the animal with the type of vitamin that we arbitrarily withheld from the diet, an appreciable number of chicks were cured, without any changes in the method of feeding, housing, or handling.

TABLE VI.  
*Ophthalmia in Chicks, Cured by Oral Treatment.*

Sample No.	Daily amount given. gm.	Chicks treated.		Description of treatment used.
		Total No.	Chick No.	
8082	0.2	3	74, 75, 134	Alcohol-acetone extract of alfalfa.
8082	0.1	2	192, 224	Alcohol-acetone extract of alfalfa.
7672	0.2	5	88, 89, 91, 108, 111	Alcohol-acetone extract of senna.
7942	0.1	2	90, 102	Acetone extract of alfalfa.
8172	0.1	1	99	Alcoholic potash saponification, then ether extract of alfalfa.
8172	0.05	2	115, 116	Alcoholic potash saponification, then ether extract of alfalfa.
8542	0.05	4	147, 151, 152, 153	Alcoholic soda saponification, then ether extract of alfalfa.
8552	0.05	2	155, 160	Benzine-alcohol extract of alfalfa.
9672	0.03	2	241, 271	Benzine extract of alfalfa.
8562	0.015	2	150, 156	Ether-benzine extract of alfalfa.
 cc.				
9892	3.0	1	305	Emulsion of cod liver oil.*
9892	2.0	1	285	" " " " "
9642	2.0	3	144, 154, 200	" " " " "
9642	1.0	5	222, 227, 247, 248, 251	" " " " "
8022	1.0	4	154, 183 — —	" " " " "
7552	2.0	5	222, 233, 250, 272, 294	" " " " "
8152	2.0	3	139, 196, 213	" " " " "
8152	1.0	6	112, 133, 213, 215, 221, 228	" " " " "
9932	2.0	1	184	" " " " "
9932	1.0	1	296	" " " " "
9932	0.8	1	236	Codliver oil.§

Total No. of chicks cured.....56

\* Metagen emulsion, Parke, Davis and Co. product.

† Egg emulsion, Parke, Davis and Co. product.

‡ Coco-vitamine, Lilly and Co. product.

§ Lofoten cod liver oil, Parke, Davis and Co. product.

In order to give an idea as to the treatment, some of the results are given in Table VI, describing the dosage, kind of material used, and the number of the chicks. We cannot state whether we used the minimum quantity in any case. However, it is evident that the amount (gm.) of extracts was so small that it contributed nothing of value toward increasing the fat, carbohydrate, protein, or energy of the diet. The benefit derived must be attributed to the fact that substances used were all high in vitamin A.

The evidence put forth as to the curing of this disease and as to the specificity of it, compared with the results obtained with vitamin B deficient and the normal control groups, is such that there can be little doubt but that we are dealing with a dietary condition. We (17) have shown in the case of the rat that such a disease is not infectious.

Beach (16), found that the disease, which he classed as nutritional roup in poultry, cannot be transmitted. He says:

"The results of our study of this disease have led to the belief that although it frequently affects large numbers in the same flock, it is not of an infectious nature, but rather due to some nutritional disturbance. This belief was arrived at after failure to transmit the disease from diseased to healthy birds or to find any species of bacteria which appeared to be a causative factor, and success in controlling and reproducing the disease by certain methods of feedings."

From the marked similarity of the findings of Beach and ours, we are of the belief that the two conditions are the same and that the etiology of nutritional roup is based upon an absence or subminimal amount of the fat-soluble vitamins in the ration.

*Comparative Fat-Soluble Vitamin Requirements of the Pigeon, Chick, and Rat.*—That the pigeon requires vitamin B, needs no argument. That its needs for fat-soluble A vitamin are small we have abundance of evidence from our studies with some 4,000 pigeons. Among these cases, we have had birds on polished rice for several weeks in our routine testing and none of them manifested typical fat-soluble A ophthalmia, or the urates. These statements are in marked contrast with those recorded where chicks were fed polished rice (Table IV). Further, it has been our experience that the decline in weight and the time of

the onset of beri-beri were not altered by feeding pigeons a synthetic diet deficient in vitamin B by having the vitamin A present. (Chart 6.)

Besides, in feeding pigeons on a complete synthetic diet, also on one that lacks the vitamin B, and on polished rice, we found that by adding just enough of vitamin B concentrate to keep the birds on a maintenance plane, no ophthalmia appeared in the rice-fed pigeons (Chart 6).

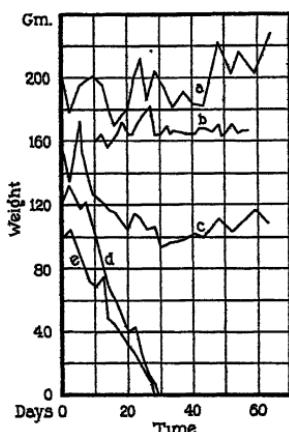


CHART 6.

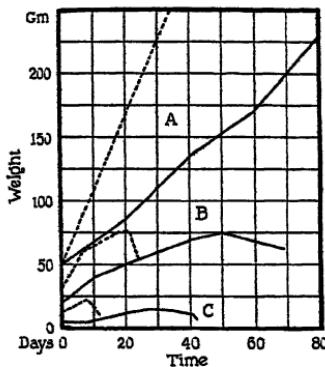


CHART 7.

CHART 6. Curve *a* represents the weight curve of pigeons fed a synthetic complete ration; Curve *b* those fed polished rice plus 0.0075 gm. daily of vitamin B preparation No. 8441; Curve *c* those fed a synthetic vitamin B deficient ration and then treated with preparation No. 5671; Curve *d* those fed the synthetic vitamin B deficient diet; and Curve *e* those fed polished rice.

CHART 7.—chicks;—rats. The rats and chicks in this experiment were fed synthetic rations made from the same purified food substances. Curve A, normal, with vitamins A and B; Curve B, vitamin A absent; Curve C, vitamin B absent.

These findings are in accord with those of Sugiura and Benedict (1); namely, that pigeons have a very low fat-soluble vitamin requirement. Whether they need none of this vitamin, as they claim, we cannot state from our data. However, as the rat requires less of vitamins A and B than the chick (Chart 7), and in turn as the pigeon seems to need less fat-soluble A than

the rat, if we can compare rice-fed pigeons when supplemented with vitamin B with rats on a synthetic vitamin A deficient diet (Charts 6 and 7 (18 to 20) it can at least be stated that the pigeons require less of this vitamin A than any of the animals thus far tested.

#### CONCLUSIONS.

From the foregoing discussion, it may be concluded:

1. Young chicks require the fat-soluble vitamin A. In its absence, the onset of the symptoms of ophthalmia appears and unless the diet is properly reinforced, or an oral treatment rich in the vitamin is given, death will eventually ensue. This ophthalmic condition has no direct relation to the absence of vitamin B.
2. The presence of urates in the tubules, kidneys, and at times on the surface of the heart, liver, and spleen is apparently related to the deficiency of the fat-soluble vitamins.
3. The eye condition, resulting from the lack of vitamin A, is evidently the same as poultry nutritional roup, described by Beach.
4. The occurrence of leg weakness in chicks fed a fat-soluble vitamin-free diet varies inversely with their age. That is, the disease is more prevalent in baby chicks 10 to 14 days old than in those over 24 days at the beginning of the experiment.
5. Young chicks and pigeons, like the rat, do not appear to require vitamin C.
6. The vitamin B requirements of young rats is less than that of chicks, and in agreement with Hart, Halpin, and Steenbock, the vitamin A requirement is also less.
7. Young and mature pigeons require very little, if any, fat-soluble vitamin A. This observation has been confirmed by Sugiura and Benedict. Our findings, however, differ from theirs in that the fat-soluble vitamin A does play a rôle in the nutrition of some species of the avian.

The authors wish to acknowledge the assistance rendered by Dr. M. J. Smead, in directing the care of the baby chicks over the preliminary feeding period, and also that of Dr. H. Preston Hoskins, in checking some of our observations.

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## THE PROPIONIC ACID FERMENTATION OF LACTOSE.\*

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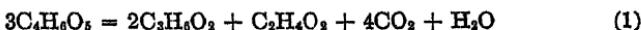
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### INTRODUCTION.

Propionic acid was one of the early products observed as resulting from bacterial action. Nearly 70 years ago (1854) Strecker (1) reported it as resulting from a fermentation of mannitol which he had studied, while Pasteur (2) also found propionic acid as one of the products formed in the fermentation of calcium tartrate. As these early observations were not made upon pure cultures, it is impossible to draw from them any definite conclusions concerning the mechanism by which the propionic acid was derived, or the organisms were involved.

In his work on microbial fermentation Fitz (3) devoted considerable study to the formation of propionic acid. Among these he noted fermentations of malic and lactic acids from which propionic acid resulted as the chief by-product. From his experimental data Fitz conceived these fermentations as proceeding according to the equations:



Malic. Propionic. Acetic.



Lactic. Propionic. Acetic.

After Fitz, no attention was given to propionic acid fermentations until 1904 when Jensen (4) discovered this acid to be one of the prominent products developed in Emmenthal cheese during the curing process. Von Freudenreich and Jensen (5) then succeeded in isolating from cheese, bacteria which produced propionic and acetic acids from calcium lactate, apparently in accordance

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with the formula of Fitz. They also found lactose to undergo a propionic fermentation which gave rise to the same end-products.

Effront (6) has studied the production of propionic acid by the fermentation of distillery mash with impure cultures, the propionic acid arising in this case through the transformation of nitrogenous compounds. This is an entirely different type of fermentation from the one in which we are interested, and, so far as the United States are concerned, it can have no industrial value.

Nothing has been done on the commercial possibilities of the propionic acid fermentation of carbohydrates and organic acids. Since quantitative studies of the action of pure cultures upon known and relatively pure substrates are wanting, little is known of the mechanism of such fermentations or of the factors which influence them.

#### EXPERIMENTAL.

In the work here reported we have used for the production of propionic acid the organism, designated as *Bacterium acidi propionici* (*d*), which is concerned in the production of the characteristics peculiar to Swiss cheese (7). From our preliminary experiments, which were conducted largely to determine the most desirable conditions of growth, considerable data have been collected which are of value only for purposes of orientation and hence will not be reported in detail. Also, the fact that the presence of certain other bacteria greatly accelerates the acid production of the propionic organism (8) rendered of no practical value much of our previous work concerning the formation of propionic acid and the factors which influence it in pure culture. We shall, therefore, limit the present report to a few experiments which indicate the possibilities of the process and which can serve as a basis for further and more intensive studies.

Because of the slow growth and the anaerobic nature of the propionic organism, many of the preliminary experiments, in which light inoculations were made by the use of a platinum loop, gave varying results and in many cases extremely low yields of acid. We therefore adopted the practice of using an inoculum equal to 1 per cent of the material to be fermented. An incubation temperature of 30°C. was used throughout. Volatile acids were determined by the original and unmodified method of Duclaux.

In Table I are given the data obtained in a preliminary experiment which throws some light on the nature of the organism used, its rate of growth, and the mechanism of the process. The unbuffered medium used (1 per cent peptone, 2 per cent lactose), though obviously of no value for quantity production, served well to reveal certain points about the fermentation.

The extremely slow growth of the organism is strikingly brought out in this table. It is seen also that the propionic acid produced amounted to nearly twice the quantity of acetic acid. A determination of the lactose content of the medium at the beginning of the experiment and again after 30 days incubation showed that the weight of the propionic and acetic acids was 66.56 per cent of the weight of the sugar fermented.

TABLE I.  
*Course of the Propionic Fermentation in an Unbuffered Broth.*

Period of incubation. days	pH	Acids produced per 100 cc. of medium. gm.		Ratio of mols of propionic to mols of acetic.
		Propionic.	Acetic.	
0	7.0			
4	5.0	0.0231	0.0187	1.68
10	4.6	0.0685	0.0450	1.52
30	4.2	0.1589	0.0843	1.88

The yield of acids is, of course, greatly increased in the presence of a neutralizing substance. For example, in a representative test in which the medium consisted of 1 per cent peptone, 2 per cent lactose, and 2 per cent calcium carbonate, 0.237 gm. of propionic and 0.134 gm. of acetic acid was obtained after 30 days incubation. In this case 74.32 per cent of the lactose fermented was recovered as propionic and acetic acids. Since on the basis of the reaction of Fitz about 77 per cent of the lactose should be accounted for by these acids, the figures obtained would indicate the probable correctness of his conception of the fermentation.

Tests which were run to determine the optimum lactose concentration for the organism were disappointing in that it does not appear that the rate of the fermentation can be appreciably increased by increasing the concentration of the sugar (Table II). It is interesting to note, however, that the optimum concentration appears to be about that which is found in milk whey.

## Fermentation of Lactose

TABLE II.

*Influence of Lactose Concentration upon the Yield of the Propionic Organism in Pure Culture.*

Lactose. per cent	Incubation time. days	Volatile acids produced per 100 cc. of medium.		
		Total 0.05 N. cc.	Propionic. gm.	Acetic. gm.
1	15	3.2		
2	15	9.4		
4	15	55.2	0.1152	0.0624
8	15	72.0	0.1433	0.0810
16	15	60.0	0.1230	0.0693

In a preliminary paper published elsewhere (8) we reported that certain other organisms accelerate the activity of the propionic acid bacteria. This has been found to be true of a number of bacteria of different types: *Streptococcus lactis*, *Lactobacillus casei*, *Proteus vulgaris*, and a number of unidentified organisms, some proteolytic in their activities and others belonging to the alkali-forming group. Table III shows the effect of two of these organisms upon the propionic fermentation. The organism designated as No. 45.4 is an unidentified laboratory culture, an active alkali producer which does not ferment lactose, probably

TABLE III.

*Effect of the Associated Growth of Other Bacteria upon the Propionic Organism.*

Inoculation.	Incubation time. wks.	Acid produced per 100 cc.		Ratio of mols of pro- pionic to mols of acetic.
		Propionic. gm.	Acetic. gm.	
Propionic organism alone.....	5	0.2997	0.1350	1.9
	8	0.3589	0.1434	2.1
	12	0.3423	0.1365	2.1
Propionic organism + <i>Lactobacillus casei</i> .....	5	1.7949	0.6237	2.3
	8	1.8256	0.8326	1.8
	12	2.3720	0.9483	1.9
Propionic organism + No. 45.4.....	5	0.6951	0.2421	2.3
	8	1.1846	0.2706	3.5
	12	1.8519	0.1305	11.5

belonging to that group of organic acid-fermenting bacteria of which *Bacterium alkaligenes* is a well known representative.

*Lactobacillus casei* has, on the whole, given the greatest stimulating action of any of the organisms we have used. The action of Culture 45.4 is of interest not alone because of its stimulating action upon the propionic organism but also on account of its effect upon the ratio of acids resulting from their combined action. Although the increased ratio of propionic to acetic acid obtained in the above experiment was probably produced too slowly to be of practical value, it indicates the possibility of purifying the products of the fermentation by biological means. It is hoped that this possibility may be dealt with more in detail in a future report.

TABLE IV.

*Effect of the Associated Growth of Lactobacillus casei upon the Fermentation of Calcium Lactate by the Propionic Organism.*

Inoculation.	Incubation time.	Acid produced per 100 cc.	
		Propionic.	Acetic.
	days	gm.	gm.
Propionic organism alone.....	10	0.0874	0.0477
" " + <i>Lactobacillus casei</i> ...	10	0.5242	0.2961

Since the propionic organism is an active fermenter of lactates, it might be assumed that the increased yield of propionic and acetic acids resulting from its growth with *Lactobacillus casei* is due simply to the action of the lactic organism upon the lactose, the lactic acid, supposedly, being more available for the propionic bacteria. That this explanation is not the correct one is shown by the results of the experiment reported in Table IV. In this experiment the propionic organism and *Lactobacillus casei* were grown together in a medium composed of 2 per cent calcium lactate, 1 per cent peptone, and 0.1 per cent lactose. The small addition of lactose was necessary in order to insure a vigorous growth of *Lactobacillus casei*, but the amount used was insufficient to have an appreciable effect upon the yield of volatile acids.

The data given in Table IV, as well as the results obtained with non-lactose-fermenting organisms, show clearly that the stimu-

lating effect of these bacteria upon the activity of the propionic organism is not due to an alteration of the lactose to a more available form.

The rate of the propionic fermentation in pure and mixed cultures is indicated by the figures given in Table V. In this case only the figures for total volatile acids were determined.

Our results have indicated that it is possible to produce propionic acid from lactose in quantities which might prove of commercial value (Table III). Although it has been found possible

TABLE V.

*Rate of Formation of Volatile Acids by the Propionic Organism in Pure and in Mixed Cultures.*

Inoculation.	0.05 N volatile acids produced per 100 cc. of medium.				
	1 week. cc.	2 weeks. cc.	3 weeks. cc.	4 weeks. cc.	5 weeks. cc.
Propionic organism alone.....	32	60	72	112	126
" " + <i>Lactobacillus casei</i> .....	200	324	498	693	
Propionic organism + No. 45.4.....	24	72	120	184	269

to speed up the reaction by the use of a proper combination of cultures (Table V), the slowness of the process makes its practical utility doubtful. However, the results thus far obtained indicate that a more thorough study of the factors influencing the fermentation would probably result in information which would make possible larger yields and at a greatly increased rate of production.

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## THE DETERMINATION OF THE TITRATABLE ALKALI OF THE BLOOD WITH DINITROSALICYLIC ACID.

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Greenwald and Lewman have recently published a method (1) for the determination of the titratable alkali of the blood. They use picric acid to precipitate the blood proteins and determine the excess of picric acid in the filtrate by titrating with 0.01 N alkali, using several different indicators and obtaining several different end-points. The free and combined picric acids in the filtrate are determined either gravimetrically or by titration with titanous chloride. Subtracting the free picric acid from the total of free and combined picric acid gives a measure of the titratable alkali of the blood, which is expressed as cubic centimeters of 0.1 N alkali per 100 cc. of blood. Different results are obtained when different indicators are used; the results are lower with indicators changing color on the alkaline side of neutrality.

The method requires considerable time, for the precipitated blood should stand in contact with the picric acid for  $\frac{1}{2}$  hour in order to allow complete combination of the acid with the bases to take place, and the estimation of picric acid by precipitation with nitron (which Greenwald and Lewman prefer to the titration with titanous chloride) requires about 2 hours. If a method of this sort is to be extensively used clinically it should be shorter and simpler.

The authors believe that they have achieved this by using dinitrosalicylic acid instead of picric acid in the determination. Dinitrosalicylic acid was introduced by one of us in 1921 as a

reagent for the estimation of sugar in normal and diabetic urine (2). Investigation has shown that it is an excellent protein precipitant (under suitable conditions it can be used to detect 1 part of serum albumin in 200,000 parts of water), and a preliminary report has been made of the use of this acid in the determination of blood sugar (3). Dinitrosalicylic acid, unlike picric acid, can be determined colorimetrically. It gives an intense red color with ferric chloride, even when only small amounts of the acid are present, and this color is directly proportional to the amount present if too great an excess of the iron salt is not used. These properties of dinitrosalicylic acid indicate that it should serve well as a reagent in the determination of titratable alkali in the blood, and the authors believe that if this determination can be shortened and rendered more convenient, it should be of considerable value in the clinical study of acidosis for, as Greenwald and Lewman have shown, the amount of titratable alkali gives certain information which is not given directly by other methods of investigating the condition. In the paper given below the use of dinitrosalicylic acid in the determination is described.

#### *Preparation of Reagents.*

*3, 5-Dinitrosalicylic Acid.*—Place 1,000 gm. of pure concentrated sulfuric acid in a 2 liter flask, add 200 gm. of colorless concentrated nitric acid, mix, and cool in a pail containing finely chopped ice, salt, and water. When the acid mixture has become well chilled add, a teaspoonful at a time, rotating each time, 120 gm. of salicylic acid. The temperature of the reacting mass should be kept as low as possible and not allowed to rise above 20°C. if a pure product is to be obtained. When all the salicylic acid has been added (1 to 2 hours) brush down that which has stuck to the neck of the flask, mix well, and pour the whole into a large flask or beaker containing 4 to 5 liters of distilled water. Mix and cool. When cold filter off crystals on a Buchner funnel, using a hardened filter paper. Press down well and wash with small quantities of cold water. Dissolve the product in boiling water, filter hot, cool, and stir to prevent supersaturation. Filter and wash as before. Recrystallize once more and dry at 100°C. Yield about 140 gm.

*Dinitrosalicylic Acid Solution.*—13.2 gm. are dissolved in hot water, cooled, and made up to a volume of 1 liter.

Titrate exactly 5 cc. of this solution in a large hard glass test-tube against 0.01 N sodium hydroxide, using 3 drops of the methyl red solution, and matching the end-point against 35 cc. of the standard end-point solution described below. Add 1 drop of thymolphthalein solution and titrate to the first appearance of a green color.

*0.01 N Sodium Hydroxide.*—Keep in a well boiled out Jena flask fitted with a syphon of hard glass tubing and protected by soda-lime tubes.

*Ferric Chloride Solution.*—An approximately 10 per cent solution of ferric chloride free from turbidity was used.

*Ferric Chloride Dinitrosalicylic Standard.*—Pipette exactly 5 cc. of the 1.32 per cent dinitrosalicylic acid into a liter flask, add 50 cc. of the ferric chloride, dilute to the mark, and mix. This standard will keep for 1 week; on longer standing a precipitate forms.

*Methyl Red Solution.*—Greenwald and Lewman have prepared methyl red according to Clark (4) and have then diluted this solution ten times. We have not been able to follow these directions exactly as not all of the methyl red would dissolve and the solution was not neutral; however, the methyl red solution is usable. The directions are: Grind 100 mg. of methyl red in an agate mortar with 7.4 cc. of 0.05 N sodium hydroxide until dissolved and dilute to a volume of 25 cc. This solution is then diluted to a volume of 250 cc. It should be filtered.

*Thymolphthalein Solution.*—Dissolve 1 gm. of thymolphthalein in 100 cc. of alcohol and add 0.1 N sodium hydroxide until slightly blue.

*Methyl Red Standard End-Point.*—5 gm. of citric acid dissolved in about 600 cc. of water, 90 cc. of 1.32 per cent dinitrosalicylic acid, and 80 drops of the methyl red solution are placed in a liter volumetric flask. Sodium hydroxide is added until a satisfactory end-point is obtained, and the solution is then diluted to volume and mixed. 25 cc. of this solution are placed in a large test-tube to control the end-point when 20 cc. of the blood filtrate are titrated to methyl red. When 5 cc. of blood filtrate are titrated use 6 cc. of the end-point solution and add 1 more

drop of methyl red. 0.01 N sodium hydroxide is the only one of these reagents which must be accurately prepared. Dinitrosalicylic acid may be made as described or a satisfactory preparation may be obtained from Eimer and Amend of New York.

*The Method.*

*A. Precipitation.*—Place 2 cc. of oxalated blood in a small flask. Add 8 cc. of water and mix. Add 10 cc. of 1.32 per cent dinitrosalicylic acid with constant rotation and continue rotating for 1 minute. Filter into a test-tube.

*B. Determination of Free Dinitrosalicylic Acid.*—Pipette 5 cc. of the filtrate into a large hard glass test-tube, heat to boiling with shaking, boil for 10 to 15 seconds to expel carbon dioxide, and cool in running water. Add 1 drop of the methyl red solution and titrate with 0.01 N alkali, comparing with the standard end-point as described above. The end-point is most easily read by looking down through the tube against a white background. Add 1 drop of thymolphthalein titrate again.

*C. Determination of Free and Combined Dinitrosalicylic Acid.*—Pipette 5 cc. of the filtrate into a 100 cc. volumetric flask, add 5 cc. of the 10 per cent ferric chloride, dilute to volume, mix, and compare in colorimeter against standard.

*D. Calculation.*—Two results will be obtained, one using methyl red, the other using thymolphthalein.

Using methyl red:

<u>Reading of standard</u>	X	Cc. 0.01 N NaOH to neutralize 0.5 cc. of 1.32 per cent dinitrosalicylic acid to methyl red.	=	Value of free and combined dinitrosalicylic acid.
Value of free and combined dinitrosalicylic acid.	—	Cc. 0.01 N NaOH to neutralize 5.0 cc. of filtrate to methyl red.	—	Titratable alkali in 0.5 cc. of blood expressed as 0.01 N.

$$\begin{array}{l} \text{Titratable alkali in} \\ 0.5 \text{ cc. of blood.} \end{array} \times 20 = \begin{array}{l} \text{Titratable} \\ \text{alkali in} \\ 100 \text{ cc. of} \\ \text{blood ex-} \\ \text{pressed} \\ \text{as 0.1 N.} \end{array}$$

Using thymolphthalein, substitute titration values with thymolphthalein for titration values with methyl red in the above equations.

When 5 cc. of blood are available it is better to use this amount, and to titrate 20 cc. of the filtrate, for the percentage error involved is decreased in this way.

There are comparatively few precautions which must be observed in carrying out the determination. Greenwald and Lewman have shown that the carbon dioxide tension to which specimens are exposed previous to analysis does not effect the amount of titratable alkali found, and that the methods of drawing the blood and handling the specimens previous to analysis also do not influence the results. We have kept specimens on ice for 4 days and have found the same values as were found in duplicate samples analyzed immediately after they were taken. Determinations made immediately after filtration and upon samples of the filtrate which had stood 4 days at room temperature also agreed. Large amounts of potassium oxalate interfere with this determination as they do with the determination by the technique described by Greenwald and Lewman, but comparison between results upon defibrinated sheep's blood and the same blood to which varying amounts of oxalate had been added showed that concentrations of this salt up to five or ten times the amount needed to prevent coagulation had no effect upon the values found. This agrees with the results of Greenwald and Lewman. Table I shows that lactic acid added to blood is recovered in a satisfactory manner. Table II shows that the same results are obtained when the protein is filtered off at once, and when the solution stands 30 minutes before it is filtered. The table also illustrates the agreement found between duplicate determinations.

In Table III is given a series of values obtained on human bloods from patients suffering from various diseases. In each

experiment the clinical diagnosis and the carbon dioxide-combining capacity of the plasma (determined by the method of Van Slyke and Cullen (5)) are given. When other quantitative data were available they have been included also. Sugar determinations were made by the method of Benedict (6), urea determinations by the method of Van Slyke and Cullen (7), and the total nitrogen and creatinine determinations by the method of Folin and Wu (8).

TABLE I.

*Titratable Alkali of Sheep's Blood after Adding Lactic Acid.*

Tenth normal alkali per 100 cc. blood.			
With methyl red.		With thymolphthalein.	
Actual.	Theoretical.	Actual.	Theoretical.
cc.	cc.	cc.	cc.
49.2		36.0	
40.6	40.7	29.6	27.5
33.0	32.2	21.0	18.6
26.6	23.6	10.4	10.0

TABLE II.

*Titratable Alkali of Sheep's Blood as Influenced by Standing with the Reagent.*

	Tenth normal.	
	Methyl red.	Thymol-phthalein.
	cc.	cc.
Rotated 1 min.....	45.8	34.8
	45.4	34.8
" and stood 30 min.....	45.4	34.6
	45.2	35.8

Table III shows that the amounts of titratable alkali found are roughly parallel to the plasma carbon dioxide-combining capacity in untreated cases. The parallelism was also fair in the case of diabetes treated with iletin (except for the determination on the last day recorded), and the results agreed approximately with those found on untreated cases. In the case suffering from renal calculus with retention the amounts of titratable alkali present when alkali therapy was given were, as a

rule, relatively higher than were the values of the carbon dioxide-combining capacity of the plasma. The difference between the titration to the methyl red and thymolphthalein end-points was also greater in this case than in most others studied. The authors believe that these findings are due to the retention by

TABLE III.

Date.	Alka- line reserve. Plasma $\text{CO}_2$ capa- city.  <i>vol.</i> <i>per cent</i>	Titratable base. 0.1 N NaOH per 100 cc. to		Other blood findings per 100 cc.				Remarks.
		Methyl red.	Thymol- phthalein.	Urea N.	Total N.	Creatinine.	Sugar.	
1923								
Feb. 23	42.5	26.8	6.4	65.1	111			Septicemia; gan- grene of lung.
Mar. 23	46.5	46.4	28.4	15.4				Arteriosclerosis.
" 16	48.1	42.0	26.0					Diabetes.
Apr. 2	48.4	42.0	40.0	14.7				Hypertension; chronic nephritis.
Feb. 23	49.0	49.0	36.4	9.8				Hypertension.
Mar. 31	50.0	46.0	36.0		34.4			" (slight).
" 23	50.3	43.2	34.0					Diabetes.
Apr. 11	51.9	62.0	32.0					Psychasthenia.
" 12	51.9	50.0	42.0	21.7				Chronic nephritis; hypertension.
Mar. 19	53.8	46.0	43.0	7.0				Arteriosclerosis; slight chronic nephritis.
" 10	55.0	42.4	32.4	14.7				Essential hyperten- sion.
" 31	55.1	43.0	28.0	15.4	36.6			Hypertension; chron- ic nephritis.
" 15	55.7	48.0	38.0	14.0				Chronic nephritis (mild); cystitis.
" 21	55.7	48.0	36.0	11.1	30.3			Hypertension.
" 31	57.9	38.0	20.0	10.5	30.3			Migraine.
" 23	59.7	52.0	40.0	9.1	30.3			Hypertension; mor- phinism.
Apr. 5	62.5	48.0	32.0	16.8		1.9	145	Chronic nephritis; chronic myocarditis.
" 4	65.4	46.0	32.0	15.4			133	Fatigue.
Mar. 10	67.3	44.4	35.1					Chronic tonsilitis.

## Titratable Alkali of Blood

TABLE III—Concluded.

Date.	Alka- line reserve. Plasma CO <sub>2</sub> capac- ity.	Titratable base. 0.1 N NaOH per 100 cc. to		Other blood findings per 100 cc.				Remarks.
		Methyl red	Thymolph- thalein.	Urea N.	Total N.	Creatinine.	Sugar.	
1923	vol. per cent	cc.	mg.	mg.	mg.	mg.	mg.	
Mar. 10	10.0	5.6	Negative.	95.9			135	Double renal stone.
" 13	24.2	26.0	8.0	119		10.9		Patient received so- dium bicarbonate after Mar. 10.
" 19	33.8	66.0	42.0	98		9.2		
Apr. 2	43.3	28.0	2.0	65.1		8.0		
" 9	43.3	46.0	32.0	63.0		7.7		
								Diabetes; iletin after first determination.
" 9	15.7	24.0	10.0			364	4 p.m.	
		18.3	31.0	27.0		133	7 "	
Apr. 10	21.6	34.0	22.0			216	Before breakfast.	
		25.5	27.0	18.0		111	5 p.m.	
Apr. 11	31.3	34.0	22.0			233	Before breakfast.	
" 12	46.2	44.0	34.0			222	" "	
" 14	72.0	54.0	34.0			145	" "	
" 16	59.8	46.0	32.0			235	" "	
" 19	72.9	46.0	42.0			125	4 p.m.	
" 24	52.8	28.0	14.0			234	Before breakfast.	

All determinations except the carbon dioxide-combining capacity of the plasma were carried out upon the whole blood. Under methyl red is given the titration value to the methyl red end-point, and under thymolphthalein the titration value to thymolphthalein. Blood for all determinations was collected before breakfast except in the cases noted.

this patient of salts of weak acids. The study of the degree of acidosis in this case was made to determine the degree of operative risk, and the authors feel that the carbon dioxide-combining capacity of the plasma served as a better method for this purpose than did the determination of the amount of titratable alkali present.

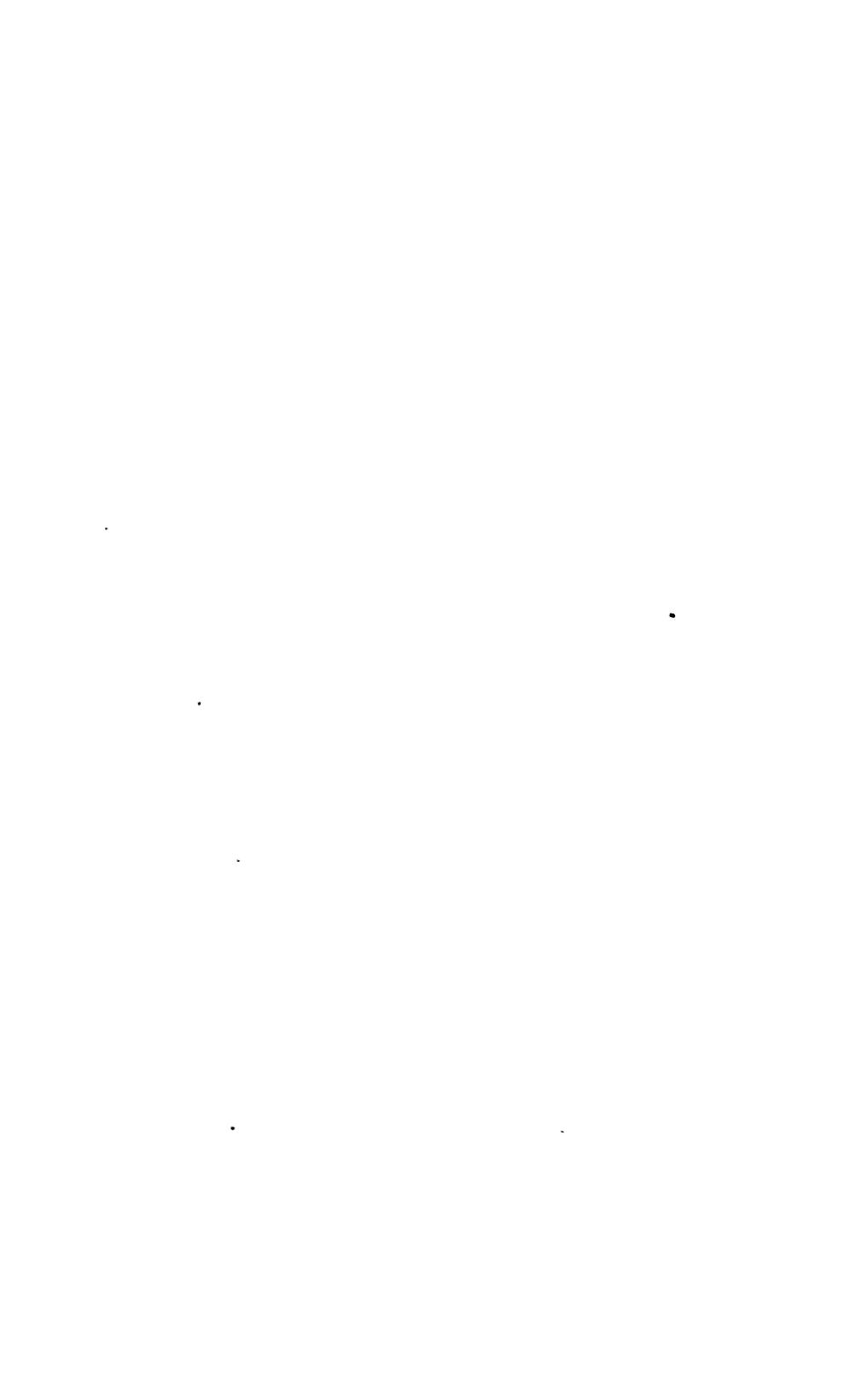
## SUMMARY.

In the paper presented a modification of the Greenwald and Lewman method for the determination of titratable alkali of

the blood is described. The method is simple and rapid enough for use in clinical laboratories. A table of results upon different types of cases is given in which a comparison is made between the titratable alkali content of the blood and the carbon dioxide-combining capacity of the plasma. In general the two methods gave results which were roughly parallel, but in a case which showed retention and which was receiving alkali therapy the amount of titratable alkali was increased by the treatment out of proportion to the increase in the carbon dioxide-combining capacity of the plasma.

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## THE FATTY ACIDS OF BLOOD PLASMA.

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In the course of work on a method for the determination of fatty acids in small amounts of blood plasma (1) certain peculiarities in the behavior of the fatty acids were noted which indicated that there was present in the mixture a considerable amount of fatty acids other than the more commonly known ones. A review of the literature showed that very little was definitely known regarding the nature and properties of the fatty acids of blood although considerable work has been done with regard to their gross amounts in different animals and under different conditions. In diabetic lipemia Fischer (2) found that the fatty acids of the blood had an iodine number of 60.6, Neisser and Derlin (3) that the fatty acids had a melting point of 35–41°C. and an iodine number of 54, and Imrie (4) that the blood fatty acids had an iodine number of 73 while those of the heart in the same individual had an iodine number of 132, the kidney 105, and the liver 104. Boggs and Morris (5), examining the blood of rabbits made lipemic by repeated bleeding, found that the fatty acids had an iodine number of from 105 to 134. Csonka (6) made a study of the fatty acids of normal human blood (whole), separating the solid from the liquid fatty acids by utilizing the difference in solubility of their lead salts in ether. He found that the liquid fatty acids of normal blood amounted to about 48 per cent of the total and had an average iodine number of about 87 varying from 76 to 105, indicating the presence in some cases of liquid acids with a higher iodine number than oleic acid. In pathological cases the percentage and also the iodine number of the liquid fatty acids were generally higher.

In view of the gaps in our knowledge regarding the nature and properties of these important constituents of blood the following study was undertaken. Most of the work was done with the blood of the sheep, cow, and pig since the blood of these animals was available in large amounts, but a few results on dog blood are presented. In the case of beef blood each sample analyzed was from a separate animal, while the samples from other animals were mixed. Attention was confined to plasma, since earlier work on lipoids of blood indicated that the lipoid content of the corpuscles remained fairly constant and independent of changes in the lipoid content of plasma with the single exception of the period of fat absorption from the intestine (7 to 9). All blood samples were from animals which had had no food for 24 hours.

#### EXPERIMENTAL.

In the work described below amounts of fresh blood plasma ordinarily of 500 cc. were used and these were treated in general as follows: To the blood in a 1 liter Erlenmeyer flask were added 80 to 100 gm. of stick sodium hydroxide and after solution of the hydroxide the mixture was digested at about 90°C. for 8 hours. The flasks were loosely stoppered during the digestion. At the end of the period the solutions were cooled and made acid to litmus by careful addition of concentrated hydrochloric acid. There was much evolution of gas—largely hydrogen sulfide—during the neutralization with tendency to foam over, especially toward the end of the neutralization. After neutrality was reached 10 cc. more of the fatty acid were added and the flask was well shaken and cooled. The mixture was then extracted in a large separatory funnel with successive portions (equal volumes) of ether until the solvent came away colorless. The extracts were united, washed with water to remove the hydrochloric acid, the ether was distilled off, and the residue dried for 1 hour at about 90°C. (water bath temperature). No effort was made at this time to avoid loss of volatile fatty acids which are undoubtedly present, but which require a special technique for their examination. To the dried residue were added 150 cc. of pentane, the flask was rotated to free the lipoid material from the bottom of the flask and to allow penetration by the solvent, and the whole allowed to stand over night with the flask loosely stoppered. In the morning

the solvent containing the fatty acids and cholesterol could generally be poured off from the considerable residue but occasionally must be filtered. The solvent together with washings of the residue was transferred quantitatively to a weighed flask, the liquid distilled off, and the residue dried for 2 hours on the water bath, then cooled, and weighed. This gives the total lipoids (A)—fatty acids and unsaponifiable matter, together with certain unknown substances which separate later with the fatty acid fraction.

The unsaponifiable fraction (largely cholesterol) was separated from the fatty acid fraction as follows: The material in the weighed flask was dissolved in successive portions of pentane (75 cc. in all) and transferred to a separatory funnel. The material generally dissolved readily in the pentane, but occasionally there was a slight yellowish residue weighing a few milligrams which dissolved in the alkaline alcohol. The flask was then washed successively with 75 cc. of 0.1 N KOH in alcohol and 75 cc. of water, both of which were added to the pentane in the separatory funnel which was then stoppered and the three liquids were mixed by shaking. The pentane separated at the top, carrying with it the unsaponifiable portion and leaving the fatty acids in the alkaline alcohol-water mixture. After separation and washing of the alcohol-water with another portion of pentane, the portions of pentane were united in a weighed flask, the solvent was distilled off, and the flask with residue dried and weighed as before. The residue (D), consisting largely of cholesterol, was generally nearly pure white and well crystallized.

The alcohol-water residue containing the fatty acids was rendered slightly acid with hydrochloric acid and shaken out with three successive 50 cc. portions of pentane, the portions were united in a weighed flask, the pentane was distilled off, and the flask dried and weighed, giving the total fatty acids (C).

For the separation of the solid from the liquid fatty acids the method of Twitchell (10) based on the differences in solubility of the lead salts of the liquid and solid fatty acids in cold alcohol was used. This method was found preferable to the lead-salt-ether method not only because it gives as good or better separation of known mixtures of fatty acids and because owing to the lesser volatility of the alcohol it is more convenient to handle but also

because in the case of the fatty acid mixture from blood it allows the separation of certain dark colored pitchy substances whose lead salts are insoluble in hot or cold alcohol but readily soluble in cold ether. The Twitchell method was found to give a good separation in all but a few samples of fatty acids from sheep plasma. In these cases repeated treatment failed to remove all the solid fatty acids, which crystallized out from the liquid residue on standing. This is believed to be responsible for the lower iodine numbers of the liquid fatty acids from sheep plasma. The Twitchell method was applied to the fatty acid residues as follows: The fatty acid fraction was dissolved in 20 cc. of hot alcohol and added to 20 cc. of a boiling cold-saturated solution of lead acetate in alcohol, the flask containing the fatty acids being rinsed out with 5 cc. of hot alcohol which was added to the main solution. The mixture was kept at the boiling point for 1 minute, allowed to cool slowly to room temperature, and let stand over night. In the morning the solvent was decanted or filtered on a hardened filter paper, from the precipitated material in the flask which was then washed twice with cold alcohol, decanting if the precipitate allowed or otherwise filtering. The filtrate should give a test for lead, otherwise the process must be repeated using more of the alcoholic lead acetate solution. The precipitate in the flask was then dissolved by boiling with 40 cc. of alcohol containing 0.25 cc. of glacial acetic acid and the hot solution, if clear, decanted from the pitchy residue and, if not clear, filtered into another flask which was then allowed to cool to room temperature and let stand over night as before. The undissolved pitchy residue in the flask was extracted twice with 5 cc. portions of boiling alcohol and these washings were added to the filtrate. Next day the liquid was decanted or filtered from the precipitate of lead salts which were then washed with cold alcohol and the washings and filtrate added to the filtrate of the day before.

The purified precipitate consisting of the lead salts of the solid fatty acids was treated with dilute nitric acid to set free the fatty acids which were extracted with ether, the ethereal solution was washed once with water, separated, the ethereal solution transferred to a weighed flask, the ether evaporated, and the residue dried and weighed (F).

The combined alcoholic filtrates containing the lead salts of the liquid fatty acids were evaporated free of alcohol, warmed with dilute hydrochloric acid to set free the fatty acids which were then extracted by shaking with pentane, the solvent was filtered into a weighed flask, evaporated, and the residue dried and weighed, giving the values for the liquid fatty acids (G).

Determinations of melting point and solidifying point were made on the mixed fatty acids before separation and on the solid fatty acids. Iodine number determinations were made on the liquid fatty acid fraction using the Hanus method.

The data obtained are presented in Table I, the amounts being calculated to 1,000 cc. of blood plasma.

#### DISCUSSION.

*Procedure.*—As may be seen from the table, the sum (B) of the fatty acid and cholesterol (unsaponifiable) fractions was almost always lower than the "Total lipoid" (A) from which they were derived. The difference in some samples may be due in part to material in the "total lipoid" which did not dissolve on the second treatment with pentane, but in most cases solution was complete and in those samples where it was not the weight of residue did not exceed a few milligrams. The most probable source of this difference was the fatty acids of less molecular weight which because of their solubility in the alcohol-water mixture were not completely extracted by pentane. Since these fatty acids were also more or less volatile at the temperature of boiling water some would be lost during the processes of drying and their loss would reduce the weight not only of the fatty acid fraction but also of the original "total lipoid."

It will also be noted that the sum of the solid and liquid fatty acid fractions (E) as found by the Twitchell process was always less and in some samples considerably less than the fatty acid fraction (C) from which they were derived. The difference was due in large part to the presence of a dark colored substance of which the lead compound was only slightly soluble in boiling alcohol and which was rejected in the scheme of separation used. Since this material is readily soluble in cold ether it would be included in the liquid fatty acid fraction as determined by the

## Fatty Acids of Blood Plasma

TABLE I.  
Fatty Acids of Blood Plasma.

Sample.	Total Lipoid per 1,000 cc.		Patty acids per 1,000 cc. by weight.	"Unsaponifiable" per cent of total lipoid (B).	"Unsaponifiable" per 1,000 cc. by weight.	Total fatty acids.	Solid fatty acids per 1,000 cc.	Liquid fatty acids per 1,000 cc.	Total fatty acids, sum of solid and liquid.	Liquid fatty acids per cent of total fatty acids (E).	Iodine No. of liquid fatty acids.	Melting point.	Sodium-potash melting point.	Solid fatty acids.	
	A	B				F			G	H					
Pig.....	1	3.62	3.10	1.88	1.22	40	38	30	0.61	1.19	1.80	65	149	48	44
	2	1.93	1.65	0.97	0.68	40	38	34	0.29	0.63	0.92	68	129	55	52
	3	3.43	3.08	2.13	0.95	31	43	38	0.69	1.38	2.07	66	131	52	50
	4	4.23	4.30	3.00	1.30	30	39	32	0.91	2.02	2.93	69	137	55	52
	5	3.28	2.92	2.07	0.85	29	29	68	1.33	2.01	66	135	55	52	
	6	2.91	2.66	1.82	0.84	31	41	37	0.56	1.23	1.79	69	142	55	52
	7	2.73	2.58?	1.64	0.89	35			0.45	1.08	1.53	70	142	55	52
	8	3.00	2.76	1.91	0.85	31			0.51	1.29	1.80	71	132	55	51
	9	3.40	2.98	1.77	1.21	40			0.58	1.26	1.84	68	143	55	51
	10	2.65	2.48	1.44	1.04	41			0.32	1.00	1.32	76	118	55	52
	11	3.02	2.88	1.69	1.19	41			0.43	1.15	1.58	75	123	55	52
	12	3.28	3.04	1.80	1.24	40	40	35	0.40	1.03	1.43	72	120	54	51
	13	3.18	2.98	1.76	1.22	41	41	34	0.44	0.87	1.31	66	134	54	51
Average ...	3.12	2.87	1.84	1.06		36	40	34	0.53	1.09	1.65	69	133	54	51

Beef.....	1	2.92	2.69	1.53	1.16	43	37	29	0.53	0.92	1.46	63	162	54	51
	2	2.00	1.71	1.02	0.69	40	39	34	0.33	0.79	2.23	72	125	54	
	3	3.12	2.80	1.58	1.22	43	38	32							
	4	2.25	2.09	1.21	0.87	41	40	34							
	5	1.98	1.81	1.04	0.77	42	43	38	0.26	0.69	0.95	72	149	55	50
	6	3.95	3.42	1.91	1.51	44	38	33	0.27	1.35	1.62	80	149	55	52
	7	3.56	3.18	1.88	1.30	41		0.38	1.27	1.65	77	143	55	52	
	8	3.35	2.88	1.64	1.24	43	39	0.26	1.10	1.36	80	153	57	54	
	9	4.31	3.90	2.06	1.84	47	45	38	0.55	1.32	1.87	71	155	55	52
	10	3.70	3.35	1.85	1.47	44	45	38	0.35	1.17	1.52	77	148	54	52
	11	3.12	2.78	1.59	1.19	42	43	37	0.36	0.99	1.35	74	154	54	52
	12	3.00	2.60	1.47	1.13	43	43	39	0.34	0.78	1.12	70	136	54	52
	13	3.30	3.00	1.70	1.30	44	44	38	0.30	0.91	1.21	76	143	54	52
	14	1.85	1.74	1.07	0.67	38	44	41	0.26	0.45	0.71	63	153	54	51
	15	1.84	1.77	1.08	0.69	38	44	40	0.35	0.63	0.98	64	132	51	49
	16	4.00	3.45	2.00	1.45	42	40	35	0.27	1.30	1.57	82	151	55	53
Average . .		3.01	2.65	1.54	1.14	43	41	36	0.34	0.98	1.40	73	147	54	51
Sheep.....	1	1.92	1.70	1.19	0.51	30	44	38	0.24						
	2	1.78	1.73	1.22	0.51	30	45	40	0.29	0.47	0.76	62		54	52
	3	1.80	1.56	1.02	0.54	34		0.29						55	52
	4	2.41	2.09	1.27	0.82	41	42	39	0.47	0.71	1.18	60	110	55	51
	5	1.90	1.59	0.94	0.65	40	42	40	0.46	0.48	0.94	51	108	54	51
	6	2.72	2.51	1.65	0.86	34	42	36	0.57	1.04	1.61	62	133	56	53
	7	2.97	2.73	1.79	0.94	35	42	38	0.54	1.11	1.66	67	130	57	53
	8	2.64	2.34	1.56	0.78	33	41	38	0.35	0.84	1.19	70	114	56	53
	9	2.51	2.06	1.32	0.74	36	40	37	0.29	0.68	0.97	70	130	56	53

## Fatty Acids of Blood Plasma

TABLE I—Concluded.

Sample.	Total lipid per 1,000 cc.		"Unaspirinifiable", per cent of total lipid (B)		Total fatty acids."		Liquid fatty acids per 1,000 cc.		Total fatty acids, sum of solid and liquid.		Liquid fatty acids, per cent of total fatty acids (E).		Iodine No. of liquid fatty acids.		Solid fatty acids.	
	A By weight.	B By weight of fatty acids and water.	C	D	E Solidifyme point.	F Melting point.	G	H	I	J	K	L	M Solidifyme point.	N Melting point.	O	P Solidifyme point.
Sheep.....	10	2.68	2.25	1.46	0.79	35	41	37	0.32	0.78	1.10	70	120	55	52	
	11	2.34	2.43	1.52	0.91	37	42	38	0.37	0.85	1.22	70	125	55	52	
	12	2.53	2.09	1.32	0.77	37	41	37	0.28	0.72	1.00	72	125	56	53	
	13	3.14	2.60	1.60	1.00	38	40	38	0.50	0.96	1.46	66	128	57	55	
	14	2.38	2.16	1.38	0.78	35	35	31	1.00	1.31	76	110	60	58		
	15	2.26	2.16	1.38	0.78	35	35	32	1.00	1.32	75	116	60	58		
	16	2.26	2.09	1.32	0.77	36	36	33	0.95	1.28	73	116	60	58		
	17	2.96	2.76	1.74	1.02	36	36	28	1.26	1.54	80	111	60	58		
	18	2.83	2.67	1.67	1.00	37	37	35	1.28	1.63	78	109	61	59		
Average ...	2.45	2.20	1.40	0.78	36	42	38	0.36	0.88	1.26	69	118	57	54		
Dog.....	1	4.67	4.33	2.78	1.55	36	40	38	0.63	1.67	2.30	72	140	58	56	
	2	4.25	3.99	2.76	1.23	31	42	40	0.68	1.67	2.35	71	150	58	55	
	3	4.27	4.21	2.71	1.50	36	42	40	0.68	1.68	2.36	71	150	57	55	
	4	5.15	4.86	3.25	1.61	33	42	38	0.78	2.12	2.90	73	163	57	54	
	5	5.88	5.66	3.57	2.09	37	43	39	0.60	2.26	2.86	78	161	57	55	
	6	5.24	4.95	3.34	1.61	33	44	40	0.71	1.87	2.58	72	159	56	55	

lead-salt-ether method of separation. Some of this pitchy substance from sheep plasma was collected, dissolved in ether, freed from lead by shaking out with warm dilute hydrochloric acid, and an iodine number determination made on it. The value obtained was 24. The exclusion of this substance in the Twitchell separation may possibly explain the relatively high iodine numbers for plasma fatty acids reported in this paper.

Since the total lipoid (total extract soluble in pentane as obtained by weight) contained volatile fatty acid the amount of which probably varied with the treatment of the sample, another value for total lipoid (B), obtained by adding together the weights of cholesterol and fatty acid separately determined, was used in the calculation "Unsaponifiable per cent of total lipoid." Similarly in the calculation "Liquid fatty acid per cent of total fatty acid" the value (E), found by adding together the fractions of liquid and solid fatty acids, was used instead of the total fatty acid as found by weight (C) although in this case it may be questioned whether the pitchy residue of which the lead salt is insoluble in hot alcohol and which makes up the difference between the values (C) and (D) should not be included, since it seems likely that it consists of fatty acid although of a different type from the others. Its amount as shown by the difference in weight between (C) and (D) varied greatly, but appeared to be less in the samples from the pig than in those from the other animals.

Melting point determinations on the mixed fatty acids were unsatisfactory for the reason that the mixture consisted of crystals of the solid fatty acids suspended in liquid fatty acids and the melting point was the point of solution of the crystals in the liquid. The melting point as given was the lowest temperature at which solution would take place. Similarly, the solidifying point was the highest temperature at which complete crystallization of the solid fatty acids took place.

As noted above no determinations were made of volatile fatty acids nor were special precautions taken to include them or remove them from the mixture and the extent to which the volatile fatty acids influenced the values obtained is unknown. It is probable that the value "Total lipoid" includes a certain proportion of volatile fatty acids and that the difference between total lipoid by weight and total lipoid by addition of the values of unsaponifiable

matter and fatty acids may be a rough measure of the volatile fatty acid content.

*Iodine Number.*—Beyond carrying out the various separations and determinations as quickly as possible and protecting the solutions from free access of air by stoppering the containers no special precaution was taken to avoid oxidation of the unsaturated fatty acids. In most of the samples there was to be found in the flasks after exposure of the fat to the iodine solution in the iodine number determinations varying amounts of a yellowish precipitate characterized by its great insolubility in organic solvents. This probably represents iodine addition products of the more highly unsaturated fatty acids, hexaiodides and possibly octaiodides, indicating the presence of some of these acids in the mixture. The fact that such high numbers were obtained without special precautions to avoid oxidation indicates that these highly unsaturated acids may not be specially sensitive to oxidation.

*Results.*—The values for total lipoid show wide variations in different species and in individuals of the same species. Pig and beef plasma are very similar in their values while those for sheep plasma are markedly lower and the few values for dog plasma markedly higher than these two. The same may be said of the values for unsaponifiable substance. The total "unsaponifiable matter" shows a constant relation to the total lipoid in all species, averaging 36 per cent in pig, sheep, and dog, 43 per cent in beef. The method of separation tends to give high values for "unsaponifiable" at the expense of the fatty acids and it would probably be a close enough approximation to say that the value for the "unsaponifiable" fraction is one-third of the value for total lipoid and one-half that for total fatty acid.

Very similar results have been obtained for human blood plasma by the micro methods (7). Using the average figures, the "cholesterol per cent of the total lipoid" (cholesterol and fatty acid) for both men and women was found to be 37.

There is also a constant relation between the values for liquid and solid fatty acids in all species, the liquid acids averaging 69 per cent of the total fatty acids in pig and sheep and 73 in beef. Since the method of separation of solid and liquid works in favor of the liquid fraction the results indicate that the liquid fatty acids constitute about two-thirds of the total fatty acid.

The iodine numbers of the various samples of liquid fatty acid are all relatively high, considerably higher than have been previously reported for the fatty acids of blood, but since the data in the literature do not apply either to blood plasma or to the separated liquid fatty acids from blood plasma the results are hardly comparable.

The constant ratio between solid and liquid fatty acids together with the relatively constant iodine number of the liquid fatty acids and the constant melting point of the solid fatty acid fraction explains the remarkably even melting points of the mixed fatty acids of blood plasma. These melted always in the neighborhood of but slightly above body temperature and solidified again at a temperature almost always slightly below body temperature which means that if they were free in the body they would probably be liquid.

The solid fatty acids from the blood plasma of all species have, as noted above, very constant melting and solidifying points indicating a close similarity in composition. A combined sample was found to have an iodine number of 6. For further examination, material (lead salt) from many samples was collected, decolorized with blood charcoal, the lead salt recrystallized many times from alcohol, and the fatty acids were recovered. The purified material had a melting point of 55° (practically the same as the raw material—see Table I) and a molecular weight as determined by titration in chloroform with standard alcoholic alkali of 255 to 260, indicating that it consisted mainly of palmitic acid.

The outstanding features of the data obtained in this paper are: (a) the presence in blood plasma of highly unsaturated fatty acids, and (b) the constancy of composition of the lipoid mixture of blood plasma as shown by: (1) the relative constancy in iodine number of the fatty acids in all species, the lower values for sheep plasma being probably due to incomplete separation of the solid fatty acids, (2) the constancy of relationship of unsaponifiable matter to fatty acids and of solid to liquid fatty acids, and (3) the constant melting point of the fatty acid mixture and of the solid fatty acid fraction.

For our ideas regarding the significance of the unsaturated fatty acids in metabolism we are largely indebted to Leathes (11).

It is his belief that desaturation is a necessary preliminary step in the utilization of the comparatively stable fatty acids of the food or bodily stores by which they are rendered more sensitive to later changes, a sensitization rendered necessary by the very circumscribed conditions of temperature, reaction, water content, etc., characteristic of the living animal body. He was able to show that the liver was the main place where desaturation took place although unable to exclude the participation in the process of other organs like the heart and kidney which are also characterized by the presence of highly unsaturated fatty acids. The desaturated fatty acids are carried from the liver to the active organs by the blood and the presence of highly unsaturated fatty acids in the blood as found in the present work was to be expected if his hypothesis is correct.

Examination of the lipoid content of the liver and other organs under various conditions has shown that the higher the lipoid percentage the lower the iodine number (12, 13). As regards the samples of blood plasma examined this inverse relation does not hold, but if anything the opposite, since in all cases where the lipoid was high the iodine number was also high. But the lipoid values found are never very high and there is no doubt that when there is a large inflow of fat into the blood either from the food directly as in alimentary lipemia or less obviously in diabetic lipemia the relation would hold as indeed has been shown by Imrie for diabetic lipemia (4).

In view of the function of the blood as a carrier the remarkable constancy of composition and of relationship between the lipoid constituents as reported above as well as the constancy of composition of the fatty acid mixture calls for comment. The known lipoid constituents of the blood are cholesterol, cholesterol esters of the fatty acids, phospholipoids of the nature of lecithin, and, at times at least, fat. Free and combined cholesterol have been shown to bear a constant relationship to each other (14, 15) as have also cholesterol and phospholipoid (7, 16). The results of the present investigation show that similar constancy of relationship exists between "unsaponifiable substance" (mainly cholesterol) and total fatty acids and between liquid (unsaturated) and solid (saturated) fatty acids also in the nature of fatty acids as shown by their constant melting points and iodine numbers.

Taken altogether the evidence indicates that the fatty acids in (fasting) blood plasma are present in definite compounds of which the composition with respect to saturated and unsaturated fatty acids is constant and which exist in definitely balanced relation to each other. This constancy appears to hold for all species and with widely different total lipoid content of the blood.

#### SUMMARY.

The presence of highly unsaturated fatty acids in normal blood plasma is shown and its significance discussed.

The amounts of total lipoid, fatty acid, and unsaponifiable matter in blood plasma are variable in different species and to a less extent in individuals of the same species, but there is a remarkable constancy in the relationships between the fatty acids and the unsaponifiable matter and between the solid and liquid fatty acids which holds for both individuals and species. The unsaponifiable matter is about one-third of the total lipoid and the liquid fatty acid is about two-thirds of the total fatty acids in all.

The fatty acid mixture apparently consists of the same or nearly the same constituents in all samples, as shown by the closely similar melting point of the mixtures and of the solid constituent and the similar iodine numbers of the liquid fraction.

The evidence indicates that, in agreement with earlier work the fatty acids of fasting blood plasma are present in definite compounds which exist in a definitely balanced relation to each other and also that the composition of the compounds with respect to saturated and unsaturated fatty acids is quite constant through the range of species examined and over a considerable range of total lipoid values.

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## AN IMPROVED PROCEDURE FOR METABOLISM EXPERIMENTS.

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### INTRODUCTION.

There are two general types of metabolism experiments: those of relatively short duration covering several periods, each from a few days to a week in length; and those carried out over as long a period as possible. As examples of the first type we have such investigations as the study of the effect of a given substance upon the nitrogenous equilibrium, or the study of the ability of the animal body to divert urea or other excretory nitrogen for the synthesis of special conjugate radicles of interest in intermediary metabolism; other examples will doubtless suggest themselves to the reader. As illustrations of metabolism experiments extending over months rather than days may be cited the investigations of Abderhalden and his collaborators into the ability of completely digested protein to maintain nitrogenous equilibrium over long periods and to replace adequately the intact protein molecule in nutrition. Some of Abderhalden's experiments lasted over a period of 74 days.

Examination of the protocols presented in Abderhalden's paper (1912) discloses the fact that loss of the desire to eat on the part of the experimental animals presented quite an obstacle to the complete success of the experiments. This failure of appetite appeared anywhere from the 7th to the 21st day of the experiment, a fact that is striking in its similarity to the behavior of our own animals which were fed diets adequate in all respects except vitamin B (and vitamin C<sup>1</sup>). Examination of Abderhalden's diets makes it

<sup>1</sup>So far as the writer is aware, no one has demonstrated that the dog requires vitamin C; the experience of this laboratory certainly has not

appear highly probable that his animals were losing their appetites as a result of a lack of vitamin B. Other instances where loss of appetite interfered with the successful prosecution of research in metabolism experiments might be cited; it is possible that in many of these cases, failures, that were attributed to monotony of diet and other seemingly more obvious causes, were really due to an absence of vitamin B from the diets being fed. Therefore, any procedure which serves to prevent loss of the desire to eat in experimental animals seems worthy of consideration and description.

Karr (1920) observed a relation between vitamin B and the appetite in the dog; the writer's investigations (1921) have confirmed and extended his findings. Such a relationship is of peculiar interest to students of metabolism and, in view of the author's most recent experiments, may be made to render a valuable service by being used to make experimental animals eat during the periods of metabolism experiments.

In his experiments Karr used as sources of vitamin B, dried brewery yeast, dried bakery yeast, a suspension made by squeezing canned tomatoes through cheese-cloth, and a crude concentrated preparation of this vitamin made from yeast according to the directions of Osborne and Wakeman (1919). In addition to confirming his results with dried brewery yeast and tomato juice, the present writer found that alcoholic extracts of rice polishings, wheat embryo, and navy bean—all of which were demonstrated by tests on pigeons to contain vitamin B—were likewise effective in restoring the appetite to dogs which had been subsisting on a diet lacking this factor.

While these experiments were being carried on, a commercial vitamin B concentrate, prepared from yeast in accordance with the main directions of Osborne and Wakeman, became available, this material being prepared by Dr. I. F. Harris and marketed under the name "Yeast Vitamine Powder (Harris)." <sup>2</sup> Tests of this product for its content of vitamin B, as carried out in this laboratory on young rats by the Osborne and Mendel technique, demon-

shown this, some of our animals having been fed on a diet lacking this accessory for over 200 days without the appearance of any symptoms of scurvy whatever.

<sup>2</sup>Sold by the Harris Laboratories, Tuckahoe, New York.

strated it to be a highly concentrated form of vitamin B; it was therefore tested for its appetite-promoting power on dogs by the same methods that had been employed in the earlier experiments with the other products containing the water-soluble vitamin. Such tests gave positive results. Trials with different doses of this material showed a quantitative relationship to exist between the amount of vitamin B administered by means of this product, the size of the animal in terms of the body weight, and the number of days over which the appetite was restored. The details of these experiments will appear *in extenso* elsewhere.

The minimum amount of "Yeast Vitamine Powder (Harris)" required for the maintenance of appetite in a dog fed in accordance with the general conditions set up in my experiments is approximately 40 mg. per kilo of body weight per day. A more definite statement in this regard does not appear to be warranted in view of the amount of error encountered in weighing the hygroscopic powder and transferring it to a gelatin capsule, the slight differences in vitamin B requirement which may be associated with age, or sex, the possible differences of vitamin B content in various samples of the commercial product,<sup>8</sup> and certain other more or less poorly defined factors which may exert an influence on the relation, but which cannot easily be controlled—the oestral cycle for example—or which may not be discoverable until more exact methods of research in this field are possible. A very safe minimum dose of this product for students of metabolism to use, in view of the above considerations would appear to be 60 mg. per kilo of body weight per day. Tests to determine the minimum dose of a wheat embryo preparation containing vitamin B and available in large quantities are now being made following the same method employed in ascertaining the minimum dose of this yeast product.

The new procedure for metabolism experiments which the writer has to suggest utilizes his determination of the minimum dose for dogs of vitamin B in terms of the "Yeast Vitamine Powder (Harris)," when the animal is fed under certain definite conditions, together with other observations which have been made in the course of his investigations.

<sup>8</sup> Personal communication from Dr. A. H. Smith.

*The New Procedure for Metabolism Experiments.*

The first important principle upon which the new procedure is based is that *the diet for the dog shall consist of a mixture of isolated food substances* which, when fed, will supply the body with everything that is necessary for proper nutrition (except vitamin B), or which lacks only one thing (other than vitamin B); namely, the variable under consideration. The second important principle followed is that *the vitamin B shall be administered apart from the food mixture* above mentioned. It is appreciated that the feeding of a purely "synthetic" food mixture in metabolism experiments using dogs as experimental animals is a procedure for the successful accomplishment of which students of metabolism have striven, and the writer makes no claim to originality for this general idea. However, no one else, except Karr who initiated the work which the author has extended, appears to have made an extensive study of the conditions under which *artificial mixtures of foodstuffs* may be fed successfully to dogs over *long* periods.

*The Diet.*

A synthetic diet, adequate in all respects as far as this is possible, and suited for the particular problem being investigated, is devised, great care being taken to make it as perfect as possible with respect to its content of (1) protein of excellent quality,<sup>4</sup> (2) vitamin A,<sup>5</sup> (3) roughage, and (4) a suitable salt mixture. Vitamin B—and this is a very important feature of this new procedure—is supplied daily by the administration *apart* from the rest of the food of a gelatin capsule containing the requisite amount of Yeast Vitamine Powder (Harris); *i.e.*, 60 mg. per kilo of body weight per day.

*Calculation of the Diet.*—The writer's practise has been to construct the diet on a kilo of body weight basis, or to form what might be called a *kilo unit* of food. It has been his experience that dogs weighing 7 kilos and over require for the maintenance of

<sup>4</sup>This, of course, would not be done if the plan of the experiment demanded the elimination of all protein nitrogen from the diet.

<sup>5</sup>Steenbock, Nelson, and Hart have shown the dog to be sensitive to the absence of this vitamin from the diet (Steenbock, H., Nelson, E. M., and Hart, E. B., *Am. J. Physiol.*, 1921-22, lviii, 14).

body weight between 70 and 80 calories per kilo per day. Following the practise of Karr—one which has been justified by the author's experience—80 calories has been selected as the number to be desired in the kilo unit of food. Dogs weighing considerably less than 7 kilos require slightly more energy intake in order to maintain their body weight; this fact, however, does not invalidate the choice of 80 calories as a unit quantity. Any variation in energy intake from this figure due to the size of the animal will not seriously affect the intake of nitrogen and salt mixture if liberal quantities such as 0.8 gm. of the former and at least 0.2 gm. of the latter be furnished with every 80 calories or kilo unit of food.

Each kilo unit of food thus prepared furnishes (1) 80 calories, (2) 0.8 gm. of protein nitrogen,<sup>6</sup> (3) from 32 to 36 calories in the form of fat, this foodstuff in grams constituting not over 25 per cent of the whole, (4) 0.4 gm. of agar or of bone ash, (5) 0.2 gm. of salt mixture, and (6) either 7 per cent of butter fat or 2 per cent of cod liver oil as a source of vitamin A. An example of such a diet is given in Table I.

At the risk of appearing to elucidate in detail what may seem quite obvious, the following suggestions are presented for calculating the kilo unit of food. They may serve to make easy for others what has been learned by more or less laborious calculation by the author.

First, determine the amount of the protein required to furnish 0.8 gm. or the amount of nitrogen decided upon, using for this purpose the figure for nitrogen content obtained upon analysis of the material. Correct for the non-protein impurities of the sample and calculate the approximate number of calories yielded by the amount of pure protein present in the preparation which furnishes the desired quota of nitrogen. Allot from 32 to 36 calories to fat, divide the particular number selected by 9, and allot the resulting figure in grams *tentatively* to fat. With sucrose supply the remaining calories in an amount sufficient to make a total of 80. Now

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<sup>6</sup>The reason for feeding so much nitrogen with every 80 calories has been indicated. The amount of nitrogen to be given may very properly be less than this figure; however, if it is less, due consideration should be given to the amino-acid make-up of the protein in order that one or more amino-acids may not thus be made limiting factors in the diet and, therefore, factors influencing the results of the experiments. Should the metabolism of such a condition, *i. e.* where the low content of an amino-acid is a limiting factor in the diet, be the object of study, one method of approach of course would be to feed a suitable protein on a low plane of nitrogen intake.

## 730 Procedure for Metabolism Experiments

allo 0.4 gm. to agar (or bone ash) and 0.3 gm. to salt mixture.<sup>7</sup> Add the several items and thus determine the total weight in grams of the kilo unit of food. Translate all amounts to a percentage basis. If the fat proves to be high—much over 25 per cent—diminish it, making up the calories by an increased amount of sucrose. This final adjustment of fat and sucrose may prove the most laborious of all the steps in the calculation; in diminishing fat and increasing carbohydrate it is necessary to determine a new total weight of the kilo unit and to recalculate the percentages. Finally, divide the fat so that butter fat or cod liver oil form 7 or 2 per cent, respectively, of the entire food mixture.

TABLE I.  
*Kilo Unit of Casein Food.\**

	Amount.	Calories.	Percentage.
	gm.		per cent
Casein { 12.7 per cent N 81.9 per cent pure }.....	6.3	20.8	37.6
Sucrose.....	5.84	23.4	34.9
Lard.....	2.83	25.5	17.0
Butter fat.....	1.17	10.5	7.0
Bone ash.....	0.40		2.3
Salt mixture†.....	0.20		1.2
Total.....	16.74	80.2	100.0

\*This kilo unit contains 80 calories, 45 per cent of which are furnished by fat, and 0.8 gm. of N.

† See Table II.

Calories are calculated on the following basis:

1 gm. of protein yielding 4 calories.

1 " " carbohydrate " 4 "

1 " " fat " 9 "

*Sources of Materials for the Diet.—Protein.*—For metabolism experiments, where protein good in quality and sufficient in quantity is desired, casein is to be recommended. It may be obtained commercially in large quantities and has a granular texture which is almost ideal for use in making the synthetic food mixture. For most purposes, perhaps, it would not require purification and a determination of its impurities. However, in view of its low content of cystine, it would seem best to supply this protein in fairly large amounts; *i.e.*, so as to furnish 0.8 gm. of

<sup>7</sup>See foot-notes to Tables II and III.

nitrogen per kilo unit of food. For experiments in which it is desired merely to secure an approximately constant daily output of urinary nitrogen, casein should prove eminently satisfactory provided its low cystine content is not allowed to become a factor influencing the results.

Other sources of protein, which have been tried and found to be satisfactory, are extracted meat<sup>8</sup> and coagulated commercial egg albumin.<sup>9</sup> The author has tested both of these products for their content of vitamin B, using the Osborne and Mendel rat technique, and found them to be free from any appreciable amounts of this dietary essential. Since Bateman (1916) has shown that the ingestion of uncoagulated egg white in moderate amounts by the dog results in a diarrhea, the commercial egg

TABLE II.  
*Salt Mixture (Karr, 1920).\**

	gm.
Sodium chloride.....	10
Calcium lactate.....	4
Magnesium citrate.....	4
Ferric citrate .....	1
Iodine in potassium iodine (Lugol's solution).....	a few drops.

\* This salt mixture, when fed along with bone ash on the basis of 0.2 gm. and 0.4 gm., respectively, per kilo unit of food, the latter serving as a source of phosphate, has given successful results with the dog during periods lasting over 5 months.

albumin must be coagulated before being used in the food mixture.. This protein does not appear to possess any advantages over casein, when used as part of the mixture to be fed to dogs; furthermore, it is more expensive. Unless the amino-acid make-up of the egg albumin in contrast to that of casein should be one of the factors under consideration in the experiment, the writer would prefer to use casein.

There is no apparent reason why any pure protein that is well utilized and not objectionable for peculiar reasons—as is gelatin, for example—could not be used in metabolism experiments, if

<sup>8</sup>From the Valentine Meat Juice Company, Richmond, Virginia.

<sup>9</sup>This may be secured from any wholesale chemical house, or from concerns supplying the baking trade, such as the Baker's Supply Company.

the diets are constructed following the general plan presented in this paper.

*Carbohydrates.*—Perusal of the literature shows glucose, sucrose, maltose, dextrin, and starches to have been used as the carbohydrates in diets fed to dogs. Of these, in the opinion of the writer, sucrose is the most desirable. As a commercial article it is unusually pure, and it is comparatively inexpensive. Furthermore, it is granular in character in contrast to the other available carbohydrates, particularly starch and dextrin, which are used a great deal in the diets fed to rats. This physical property of sucrose is of advantage in that it gives the complete food mixture a granular rather than a powdery texture. Starch and dextrin are undesirable: they have the physical properties of fine flour and with these polysaccharides it is not easy to prepare a food mixture that possesses a texture pleasing to most dogs. A food that is powdery in character tends to become sticky when moistened with saliva; dogs, when eating such a food, behave much as do animals fed a mixture containing moderate amounts of granulated gelatin.

*Fats and Sources of Vitamin A.*—There does not appear to be any reason why lard should be supplanted in the dietary as a source of calories and as an agent with which to bind the various dry substances being used. Butter fat is to be preferred to cod liver oil as a source of vitamin A, inasmuch as experience has shown that some dogs do not like the cod liver oil; furthermore, the efficiency of the total fat as a binder is lessened when a liquid fat is used.

*Roughage.*—Abderhalden tried both bone-black and bone ash in his experiments and apparently favored the latter although pointing out that not all of his animals behaved alike as regards the type of stools eliminated when the same indigestible residue was being ingested. The efficiency of bone ash as a roughage was early emphasized by Gies (1904). In his communication recommending the use of this material Gies states that no experiments were made to determine whether any of the mineral constituents of the ash were absorbed. The experience of our laboratory has been that bone ash under *most* conditions is very satisfactory as a roughage, as far as the production of a dry stool is concerned, when used to the extent of 0.4 gm. per kilo unit of food; however, the use of this ash is attended by a higher level of urinary phosphate

indicating that the bone ash does not function entirely as an indigestible residue. Inasmuch as the absorption of earthy phosphates in the intestine is influenced by many factors, chief of which are those which act by precipitating the calcium salts, the actual amount of the ash phosphate that is absorbed may be quite variable. In many metabolism experiments the amount of phosphate absorbed in the intestine or eliminated by the kidney is of no significance, and in such cases bone ash is well suited for use as roughage. On the other hand, should it be desirable to avoid the ingestion of phosphate, agar may be employed to give bulk and proper texture to the feces, the amount of this polysaccharide to be used being the same as that of the bone ash; *i.e.*, 0.4 gm. per kilo unit of food.

*Mineral Constituents.*—The salt mixture used in the writer's experiments is the same as that employed by Karr (Table II); whether it is the most ideal in composition when used under a variety of conditions remains to be determined by definite experiment. This mixture contains no phosphate, but when used in conjunction with bone ash has given complete success with dogs in feeding experiments lasting over 5 months. It is possible that this particular mixture would not be ideal when used along with agar-agar instead of bone ash, since the latter may contribute mineral materials to supplement those supplied by this salt mixture.

The author has devised a new salt mixture (Table III), utilizing the principle of supplying in the food daily an amount of each of the various inorganic substances equal to or slightly in excess of that eliminated through the kidney by a normal organism during 24 hours, these amounts being expressed in terms of grams eliminated per kilo of body weight per day; at the same time this mixture offers to the body certain constituents in fairly normal ratios to others—sodium to potassium, for example. 0.3 gm. of this mixture per kilo unit of food contains a liberal amount of the various inorganic substances desired in the synthetic diet.

From a theoretical standpoint any salt mixture of proven worth such as those that McCollum and his collaborators (1915) have used, or that of Osborne and Mendel (1913), could be employed as part of the kilo unit of food; the amount that should be used, considering the experiments of McCollum and other students of

nutrition, would seem to be about 1.5 per cent, a figure which at any rate should be the minimum.

*Vitamin B.*—Theoretically, any concentrated form of this vitamin could be used. A product which is free from protein and which contains only relatively small amounts of any of the other foodstuffs is the most desirable for metabolism experiments. Such a product should be standardized for such use in order to reduce to a minimum the amount of material of unknown composition that is given along with the vitamin, as well as to insure the administration of a sufficient amount of the dietary essential to maintain the appetite. As indicated earlier in this paper, the "Yeast

TABLE III.  
*Salt Mixture.\**

	Per kilo.	Percentage.
	gm.	per cent
Sodium chloride.....	0.1050	38.0
Magnesium citrate.....	0.0905	32.5
KH <sub>2</sub> PO <sub>4</sub> .....	0.0336	12.2
CaHPO <sub>4</sub> ·2H <sub>2</sub> O .....	0.0215	7.8
Potassium chloride.....	0.0192	7.0
Ferric citrate.....	0.0050	1.8
Potassium iodide.....	0.0013	0.5
Total.....	0.2761	99.8

\* Where 0.4 gm. of agar-agar per kilo unit is used as a source of roughage, this salt mixture may be used on the basis of 0.3 gm. per kilo unit of food.

"Vitamine Powder (Harris)" has been standardized for this purpose by the writer's experiments, and the amount to be used has been stated. Inasmuch as this preparation contains approximately 8.33 per cent of nitrogen none of which is from protein, and a No. 00 gelatin capsule contains 20 mg. of nitrogen on an average, the animal would receive approximately 3 and 5 mg. of nitrogen from the gelatin and the Harris vitamine, respectively, per kilo of body weight per day, when vitamin B is administered by this method. This amount of nitrogen is very small; certainly that derived from protein is almost negligible.

*General Remarks Concerning the Diet.*—Karr used water as one constituent of his dietaries. After some experimenting the

writer decided to build up his kilo unit of food without the use of water, and this has been a decided improvement. When this change is made, the large amount of food prepared at one time does not develop an odor due to rancid fat; neither does it dry out. Ordinary butter, which contains considerable water and salt in inconstant amounts, should be replaced by butter fat for this reason.

#### DISCUSSION.

Caspari and Zuntz (1911), in discussing how to feed animals used in metabolism experiments, pay particular attention to the problem of maintaining the appetite and suggest the use of meat extract, bouillon, etc., as aids in surmounting this difficulty. Because dogs are carnivorous, meat is advised as the basis upon which to construct the diet and sugar is suggested as of value in improving the taste. The writer's experiments (1922) have clearly demonstrated that meat extract neither restores to nor maintains the appetite in dogs which have lost the desire to eat after subsisting upon a diet adequate except with respect to vitamin B. Furthermore, the use of meat in feeding dogs is quite unnecessary: purely artificial mixtures of isolated food substances, an illustration of which has already been given in Table I, may be fed with complete success over long periods. One dog<sup>10</sup> in this laboratory was fed a casein diet in accordance with the method described in this paper with no failure of appetite whatever over a period of 150 days—approximately 5 months. Five other animals have been fed synthetic diets for 3 months and over with complete success.

These results demonstrate that monotony of diet is not *per se* the cause of failure of appetite; loss of the desire to eat is rather an expression either (1) of the failure of the food being fed to nourish the animal properly, or (2) of an adjustment of the dog to its energy requirement when offered more than is necessary of a food mixture that is adequate in all respects. Rubner<sup>11</sup> says that many

<sup>10</sup>This animal was being used by Dr. H. J. Deuel, Jr., in a study of the metabolism of pyrimidines.

<sup>11</sup>Rubner, M., Die Gesetze des Energieverbrauches bei Ernährung, Leipzig, 1902, 83; quoted by Lusk, G., The elements of the science of nutrition, Philadelphia, 2nd edition, 1909, 218.

years of experience with dogs leads him to believe that appetite and capacity for digestion and absorption depend on the dog's requirement for energy in his given state of nutrition. A diet which a dog will greedily devour when in a room at a temperature of 0°, he will in part refuse when at a temperature of 33°. The writer has secured evidence supporting Rubner's general contention and has therefore adopted, as a control of the energy factor influencing loss of appetite, the plan of feeding *only enough calories to permit maintenance in adult animals or sufficient to allow a slight increase in body weight in growing dogs.* This procedure has proved successful in all cases: its importance should not be overlooked by those who wish to make their experimental animals eat voluntarily every bit of food offered during the course of metabolism experiments.

When a dog has lost its appetite due to a lack of vitamin B, the desire to eat may be restored within 1 or 2 days by giving to the animal a single large dose of this missing dietary essential; where daily doses, each containing little more than the minimum daily requirement of the vitamin are administered, several days of irregular food intake may elapse before the dog will consume immediately all the food offered. This fact should be considered when interpreting the results obtained by supplying the vitamin to any given animal.

A few words of caution should be given here regarding certain false ideas which the demonstrated relationship between appetite and vitamin B may seem to suggest. It does not follow from the work that has been done along this line, that the administration of vitamin B will restore the appetite to all animals which have lost the desire to eat. Various pathological conditions other than vitamin B deficiency may bring about a loss of appetite; in such cases it does not seem reasonable to suppose that the administration of this vitamin will restore the desire to eat; whether it will do so, however, can only be determined by definite experiments planned to answer this question.

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## ON GLYCOLYSIS IN DIABETIC AND NON-DIABETIC BLOOD.

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The phenomenon of glycolysis has been a source of interest from the time of Claude Bernard (1) to the present day (2), and as a result many phases of the subject have been investigated and reinvestigated from various points of view. One of the most popular themes has hinged on the subject of a possible difference in the glycolytic power of normal and of diabetic blood. From the work of Lépine (3) and his pupils it would seem that diabetic blood is capable of less active glycolysis than is the blood of non-diabetic individuals; a point of view which has, however, not gained acceptance at the hands of more modern workers in this field (4). The analytical technique for the quantitative determination of blood sugar has undergone such radical revision and improvement during the past few years that we have felt justified in reopening this rather well cultivated field; in short we have again measured the rate of disappearance *in vitro* of reducing bodies from the blood of diabetic and non-diabetic hospital patients and of normal men. All determinations were made by the blood sugar method of Folin and Wu (5). We have in most cases made determinations of reducing bodies in the blood within 5 minutes after withdrawal from the body, and again after 3, 6, 24, and in a few cases 48 hours had elapsed. During the period of storage our blood samples have been kept at temperatures varying from 19 to 22°C.; in other words, at "room temperature." Our reason for using this procedure instead of resorting to the more constant temperature furnished by a refrigerator or an incubator is that it was necessary to transport the blood from the hospital to the laboratory, a distance of several miles, and we have therefore made use of what

## Glycolysis in Blood

happened to be the outdoor temperature of the days on which the experiments were carried out.

TABLE I.

*Showing the Rate of Glycolysis in the Blood on Non-Diabetic Subjects.*

No.	Diagnosis.	Glucose per 100 cc. blood.				Loss.		
		At once.		3 hrs.	6 hrs.	24 hrs.	3 hrs.	6 hrs.
		mg.	mg.	mg.	mg.	per cent	per cent	per cent
1	Syphilis.	97	80	73	33	7	24	65
2	Chronic nephritis.	121	100	95	72	17	21	60
3	Gastric ulcer.	142	105	90	88	26	36	62
4	Cardiac.	137	115	110	68	16	19	50.4
5	Normal.	105	88	75	43	16	23	60.0
6	"	102	86	81	49	15	20	52
7	"	108	100	88	41	7	18	62
8	"	117	108	93	59	7	20	50
9	"	105	88	80	41	16	23	60
10	Chronic cholecystitis.	100	87	71	43	13	29	57
11	Normal.	90	80	71	41	11	21	54
12	Arteriosclerosis.	100	95	80	43	5	20	57
13	Normal.	83		60	33		26	60
14	"	90	80	71	51	11	21	43
15	"	100	87	71	43	13	29	57
16	"	117	100	91	70	14	22	40
17	"	111	90	83	57	18	25	49
18	"	103	102		44	0.9		57
19	"	102	95	80	40	6	21	60
20	"	107	95	81	50	11	24	58
21	"	110	95	86	60	13	21	55
22	Jaundice.	107	97	75	33	9	30	69
23	Nephritis.	100	88	72	32	12	28	68
24	Normal.	107	102	74	33	4.6	31	59
25	Syphilis.	114	105	87	47	7	23	56
26	Normal.	87	74	71	32	14	18	63
27	Prostatitis.	111	100	87	33	9.9	21	70
28	Normal.	90	77	69	33	13	21	60
29	Hyperthyroid disease.	108	95	83	30	12	23	72
30	Renal diabetes.	100	66	64	30	33	36	70

In Table I are collected the results obtained on the blood of thirty non-diabetic individuals, some of whom might be classed as in strictly normal physical condition, and some of whom were

distinctly pathological. Table II contains results on blood taken from thirty cases of diabetes, which were selected to give examples

TABLE II.  
*Showing the Rate of Glycolysis in Diabetic Blood.*

Speci- men No.	Glucose per 100 cc. blood.				Loss.			Remarks.
	At once.	After 3 hrs.	After 6 hrs.	After 24 hrs.	After 3 hrs.	After 6 hrs.	After 24 hrs.	
	mg.	mg.	mg.	mg.	per cent	per cent	per cent	
1	620			590			4	In coma, died next day.
2	665			660			4	Same patient as above. Died 3 hrs. later.
3	274	267		260	2.5		5	In coma, died 30 min. later.
4	132	130	128	122	1.5	3.0	7.5	
5	634	606	606	570	4	4	10	
6	120	120	120	108	0	0	10	
7	250	250		222	0		11	
8	440			380			13.6	
9	392	392	380	336	0	3	14	
10	222			200			9	
11	332			282			15	
12	121	105		102	12		15	
13	400	400	380	332	0	5	16	
14	162			133			17	
15	176	162	156	143	7	11	18	
16	444	444	410	348	0	7	19	
17	280	266	260	216	5	7	22	
18	234	216	216	180	7	7	23	
19	117	103	110	83	11	14	24	
20	346		306	266		11	25	
21	210	210	206	156	0	1.9	25	
22	238			172			27	
23	222	198	198	158	1.8	1.8	28	
24	113	103	91	80	8	18	29	
25	121			86			29	
26	332	300	290	234			29	
27	400	380	360	280	5	7	30	
28	258	246	234	173	4.7	9	32	
29	210		172	144		18	32	
30	138	130	114	83	5.8	17.4	32	

of all ordinary grades of this disease, ranging from extremely severe to very light.

In the blood of the non-diabetic individuals the percentage loss in reducing bodies in 24 hours varied from 72 to 40 per cent, in 6 hours from 36 to 19 per cent, and in 3 hours from 22 to 6 per cent. In contrast to the above figures are those collected in Table II which consists of the results obtained on diabetic blood. Here it will be seen that a much smaller percentage loss of reducing bodies is observed; for the 24 hour period the losses vary from 32 to 4 per cent, in 6 hours from 17 to 0 per cent, and in 3 hours from 11 to 0 per cent.

Our results are of interest from two points of view. In the first place they indicate the relatively large percentage loss of blood sugar which may sometimes result from the all too common practice of allowing samples of blood to stand for several hours before precipitation is carried out,<sup>1</sup> and in the second they may be regarded as confirming the observations of Lépine regarding the difference in glycolysis of diabetic and non-diabetic blood. Lépine and his followers maintained that the characteristic symptoms of diabetes were due to a lack or scarcity of the proper enzymes needed to produce glycolysis, a point of view which never gained general acceptance even in France, and which is but poorly supported by experimental or clinical evidence. In a recent paper Winter and Smith (6) present experimental evidence which suggests that the blood sugar of normal individuals is  $\gamma$ -glucose, whereas the blood sugar of persons suffering from a severe grade of diabetes mellitus is of an abnormal nature and is probably  $\alpha\beta$ -glucose or possibly a polysaccharide. These investigators postulate the existence of an enzyme capable of transforming the  $\alpha\beta$  form of glucose into the  $\gamma$  form, but state that such an enzyme is absent from blood and must therefore be furnished by some other tissue. The work of Hewitt and Pryde (7) would suggest that such an enzyme exists in the mucous membrane of the intestine, an enzyme which appears to be capable of transforming both glucose and fructose into  $\gamma$ -glucose, but which is capable of effecting these transformations *in vivo*, only; and Winter and Smith (8) have recently published the results of experiments which indicate that an enzyme of this nature also exists in the liver and can produce its characteristic effects *in vitro* when activated with extracts of pancreatic tissue.

<sup>1</sup> Data bearing on this point have also recently been published by Birchard (Birchard, D. E., *J. Lab. and Clin. Med.*, 1923, viii, 346).

The work of Winter and Smith is of great value in furnishing ground for a hypothesis regarding the reason for the differences in the rate of glycolysis in diabetic and non-diabetic bloods. It is easily conceivable that in non-diabetic blood in which the blood sugar is all in the form of  $\gamma$ -glucose the glycolytic enzyme capable of attacking this form of glucose is supplied with the substrate suitable for the production of the maximum result; in moderately severe diabetes where we may consider the possibility of the existence of a mixture of  $\alpha$ ,  $\beta$  and  $\gamma$  forms the glycolytic action is less because the enzyme is presumably without power to attack  $\alpha$ ,  $\beta$ -glucose, whereas in extremely severe diabetes, where most of the sugar exists in the  $\alpha$ ,  $\beta$  form with probably only a trace of the  $\gamma$  form present, we get very slight or sometimes practically no glycolytic action. As an example of this latter condition we wish to call attention to Specimens 1 and 2 (Table II), which were taken from a woman, in coma, who died 24 hours after Specimen 1 was taken and 8 hours after the removal of Specimen 2. A similar case is that illustrated by Specimen 3, which was taken from a child, in coma, who died about 30 minutes after the removal of this blood. In both of these cases glycolysis was practically negligible, as the total loss of reducing bodies found after the blood had stood for 24 hours amounted to only 4 to 5 per cent.

The amount of glycolysis appears to be in no way dependent on the blood sugar level, specimens of blood having a glucose content of 600 mg. sometimes show practically the same rate of glycolysis as those containing 125 mg., a point which may be regarded as suggesting the fact that in diabetes the qualitative composition of the blood sugar may be entirely independent of its quantitative relations.

In a series of observations which will be published shortly we have found that on successful treatment the glycolytic power of diabetic blood increases and in a few very favorable cases may approach the values found in non-diabetic individuals, a finding which may easily be explained on the assumption, that with proper treatment the blood sugar of the diabetic becomes not only quantitatively, but also qualitatively more nearly like that of the normal subject.

In conclusion we wish to acknowledge our indebtedness to Drs. I. I. Lemann and Allan Eustis who have furnished us with many of the samples of diabetic blood used in this work.

**SUMMARY.**

It has been found that glycolysis in normal blood is much more active than in the blood of persons suffering from fairly severe diabetes. In the blood of two persons dying from diabetic coma practically no glycolysis took place even after standing for 24 hours. These findings are explained on the assumption that in normal subjects the "blood sugar" consists of  $\gamma$ -glucose which is readily attacked by the glycolytic enzyme, while in persons suffering from severe diabetes probably only a small portion of the reducing bodies consist of  $\gamma$ -glucose, the main portion being the  $\alpha\beta$  form which the glycolytic enzyme is powerless to attack.

The amount of glycolysis obtained bears no relation to the concentration of the blood sugar.

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## STUDIES OF THE CARBON DIOXIDE ABSORPTION CURVE OF HUMAN BLOOD.

### III. A FURTHER DISCUSSION OF THE FORM OF THE ABSORPTION CURVE PLOTTED LOGARITHMICALLY, WITH A CON- VENIENT TYPE OF INTERPOLATION CHART.

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In the second paper of this series (1) the form of the  $\text{CO}_2$  absorption curve of blood was discussed, with special reference to the shape of the curve when plotted as pH against  $[\text{BHCO}_3]$ . Van Slyke, Austin, and Cullen (2) and Warburg (3) have shown that the curve, plotted in this way, approximates a straight line. This relation was tested on a series of twenty-six absorption curves to see how closely it agreed with the results of experimental observation. The results of this analysis indicated that the relation was only an approximation and one that probably would not hold over wide ranges of  $\text{CO}_2$  tension. It was also pointed out that curves plotted as  $\log [\text{H}_2\text{CO}_3]$  against  $\log [\text{BHCO}_3]$  were more nearly straight lines. This was true not only of our series of curves, but also applied to the whole blood curves published by Van Slyke, Austin, and Cullen (2).

The comparison of the two methods of drawing curves has been extended to other available data and in all instances the purely logarithmic straight line relation has been found to agree with observed data better than does the pH-[ $\text{BHCO}_3$ ] relation. This is especially true at low  $\text{CO}_2$  tensions. Among the data thus studied are several horse blood curves obtained in the course of the work which formed the basis of the article published by Austin, Cullen, Hastings, McLean, Peters, and Van Slyke (4), four human blood curves from Barr, Himwich, and Green (5), and a five-point curve of our own, recently determined. A less

careful analysis has been made of the curves published by Joffe and Poulton (6), Hasselbalch and Warburg (7), and Warburg (3). In the latter series neither method of construction gives as good agreement with experimental data as was obtained in Van Slyke's, Barr's, and our own experiments. It is quite possible that this is due to the experimental methods employed. Joffe and Poulton's curves are constructed from blood collected at various times over a period of a year, during which time the absorption curves must have varied considerably. The accuracy of a curve thus constructed must depend on the number of determinations made. The curve of J.J. was studied most intensively and therefore should represent most closely the actual shape of the absorption curve. The average curve of the defibrinated oxygenated blood

TABLE I.  
*Curve of J. J. from Joffe and Poulton.*

CO <sub>2</sub> tension, mm.....	10	20	30	40	55	70	90	110.3
Average CO <sub>2</sub> observed, vols. per cent.....	29.2	38.0	44.8	50.1	57.3	63.1	68.0	73.8
CO <sub>2</sub> calculated from log relation, vols. per cent.....	29.4	38.5	44.9	50.1	56.8	62.5	68.4	74.1
Difference, vols. per cent.....	+0.2	+0.5	+0.1	0	-0.5	-0.6	+0.4	+0.3

of J.J. (as estimated by Warburg) was plotted as  $x = \log [\text{pCO}_2]$ ,  $y = \log [\text{CO}_2 \text{ volumes per cent}]$ . In Table I the results of the values thus obtained are compared with the values given by Warburg between 10 and 110 mm. of CO<sub>2</sub> tension. The agreement is highly satisfactory. Above 100 mm. the observed values fall away from the straight line.<sup>1</sup>

The two methods were also applied to curves of "true" and "separated" plasma. The suitable "true" plasma material available in the literature and in our own experiments was comparatively scanty; but the results were qualitatively and quantitatively similar to those obtained from the whole blood experiments. In the case of the few "separated" plasma studies which were

<sup>1</sup> In studying the relation of pH to BHCO<sub>3</sub>, a constant pK<sub>1</sub> was first assumed. It seemed possible that the introduction of proper correction values for pK<sub>1</sub> (see Paper I of this series (8)) might give more nearly straight lines. However, it proved to increase rather than to diminish the deviations from the straight lines.

analyzed there was no significant difference between the two methods. The reason for this apparent agreement will be discussed in a subsequent communication.

The amount of experimental material that has been subjected to analysis is so large as to leave little doubt that the logarithmic

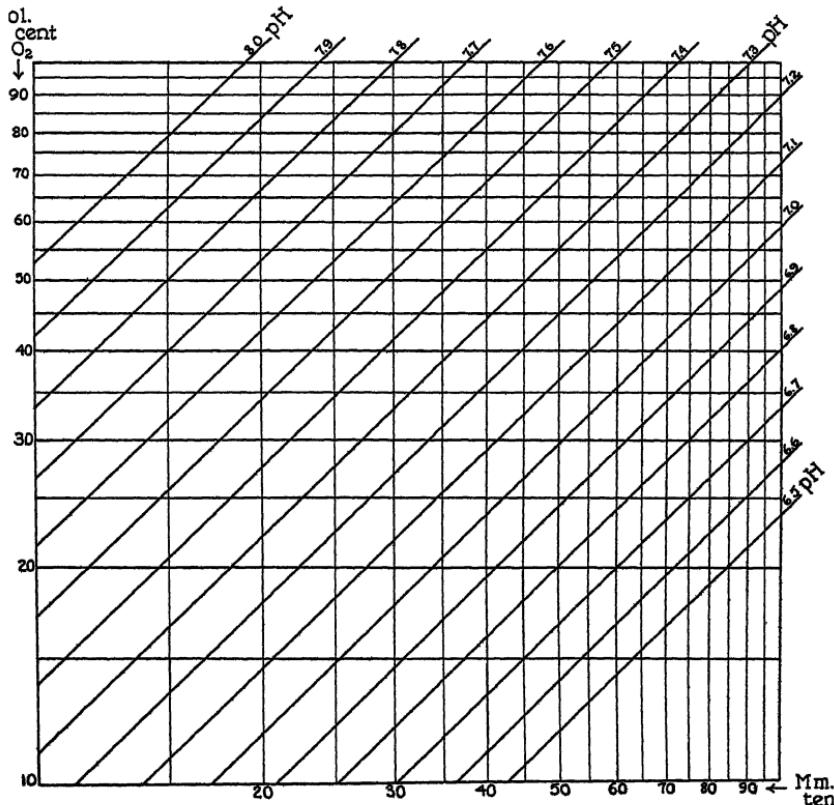


FIG. 1. Abscissa =  $\text{CO}_2$  tension in mm. of Hg; ordinate =  $\text{CO}_2$  content in volumes per cent; and the diagonal lines = pH ( $\text{pK}_1 = 6.140$ ).

relation drawn as a straight line is more nearly an expression of the facts than is the pH-[ $\text{BHCO}_3$ ] relation and holds over a wider range of  $\text{CO}_2$  tensions. It does not follow that the absorption curve actually assumes a straight line form when logarithmically plotted. The accuracy of the relation does, however,

offer a method superior to any yet proposed for the extrapolation or interpolation of points on an absorption curve. Furthermore, if the extrapolated point is not too far from one of the observed points the error of extrapolation on a curve determined by only two points will be very small.

As a general method of plotting absorption curves, however, the use of the logarithmic principle offers other obvious advantages. If the CO<sub>2</sub> tension is plotted against CO<sub>2</sub> content on logarithmic paper and pK<sub>1</sub> is constant for a given sample of blood or plasma, pH will be represented by a series of lines forming angles of 45° with the abscissa. Fig. 1 represents such a chart constructed for use with plasma at 38°C., assuming a pK<sub>1</sub> value of 6.140 (9) and a solubility coefficient of  $\alpha_{\text{CO}_2 \text{ plasma}} = 97.5 \times 0.555$ .

The chart permits the rapid visualization and calculation of changes in the three variables and introduces no curved line functions at all. Given values for any two of the three functions, pH, CO<sub>2</sub> content, and CO<sub>2</sub> tension of plasma, the third is determined. If absorption curves are plotted from two points they may also be drawn as straight lines.

#### *Method of Construction of Fig. 1.*

The Henderson-Hasselbalch equation may be written

$$\text{pH} = \text{pK}_1 + \log \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]}$$

and

$$\frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]} = \frac{[\text{CO}_2] - \frac{\alpha \text{pCO}_2}{760}}{\frac{\alpha \text{pCO}_2}{760}} = \frac{[760 \text{ CO}_2]}{\alpha \text{pCO}_2} - 1$$

where CO<sub>2</sub> = the CO<sub>2</sub> content of blood in volumes per cent,  $\alpha$  is the relative solubility coefficient of CO<sub>2</sub> in plasma, and pCO<sub>2</sub> is the CO<sub>2</sub> tension in mm. of Hg.

$$\alpha_{38^\circ} = 97.5 \times 0.555, \therefore \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]} = \frac{14.04 [\text{CO}_2]}{p_{\text{CO}_2}} - 1$$

and

$$\text{pH} = \text{pK}_1 + \log \left( \frac{14.04 [\text{CO}_2]}{p_{\text{CO}_2}} - 1 \right)$$

If  $\text{pK}_1 = 6.14$  and pH and  $p_{\text{CO}_2}$  are given,  $\text{CO}_2$  can be found by transposition and removal of logarithms.

$$[\text{CO}_2] = \frac{[\text{antilog} (\text{pH} - 6.14) + 1] p_{\text{CO}_2}}{14.04}$$

This equation may be used to locate the pH lines. Two points may be determined for each line or only one point need be determined and a  $45^\circ$  line may be drawn through this point with the aid of a protractor. The lines must be drawn with considerable accuracy because of the contraction of the scale at high values.

The same kind of chart cannot be used in the same way for studies of whole blood because the assumption of a constant  $\text{pK}_1$  is not proper in whole blood. The pH lines for whole blood will not therefore be straight. Furthermore, the  $\text{CO}_2$  solubility coefficient of blood varies according to the cellular concentration. This does not diminish the value of the method for the purpose of extrapolation or interpolation on any absorption curve.

A simple corollary to the fact that the relation of  $\log [\text{BHCO}_3]$  to  $\log [\text{H}_2\text{CO}_3]$  is more nearly a straight line than is the relation of  $[\text{BHCO}_3]$  to pH is that pH plotted against  $\log [\text{BHCO}_3]$  is more nearly a straight line. For if  $\log [\text{BHCO}_3]$  and  $\log [\text{H}_2\text{CO}_3]$  give a straight line, then

$$\log [\text{BHCO}_3] = m \log [\text{H}_2\text{CO}_3] + b$$

in which  $m$  and  $b$  are constants.

Substituting in the Henderson-Hasselbalch equation,

$$\begin{aligned} \text{pH} &= \text{pK}_1 + \log [\text{BHCO}_3] - \log [\text{H}_2\text{CO}_3] \\ \text{pH} &= \text{pK}_1 + m \log [\text{H}_2\text{CO}_3] + b - \log [\text{H}_2\text{CO}_3] \quad \text{or} \\ \text{pH} &= (m - 1) \log [\text{H}_2\text{CO}_3] + (\text{pK}_1 + b) \end{aligned}$$

which is in the form of a straight line.

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## "PERMUTIT" AS A REAGENT FOR AMINES.

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### INTRODUCTION.

In the course of chemical studies of mental disorders the need has been felt for a means of separating amines from other nitrogenous substances present in biological fluids.

The study of "permutit" as a reagent for this purpose arose from the observation made in the autumn of 1921 that adrenalin could be completely removed from solution by gentle shaking with permutit. In surveying the literature, no published articles could be found concerning the use of permutit as a reagent for amines. The studies herein reported were, therefore, undertaken with the special intention of answering two questions: (1) What kinds of substances are removed from solution by permutit? (2) In what ways can this reaction be affected by chemical and physical conditions?

Permutit is the trade name of a synthetic zeolite of the approximate composition  $2\text{SiO}_2 \cdot \text{Al}_2\text{O}_5 \cdot \text{Na}_2\text{O} \cdot 6\text{H}_2\text{O}$ , first manufactured by a German chemist, Robert Gans,<sup>1</sup> which has been used industrially for softening hard water and refining sugar. The base-trading power of zeolites and of permutit in particular, has been the subject of numerous studies, of especial interest to the physical chemist.<sup>2</sup> Folin and Bell<sup>3</sup> used permutit for the determination of urinary ammonia.

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<sup>1</sup> Gans, R., *Jahrb. K. preuss. geol. Landesanstalt u. Bergakademie*, 1905, 179; 1906, 63; cited in *Chem. Centr.*, 1906, ii, 1731.

<sup>2</sup> Ramann, E., and Spengel, A., *Z. anorg. u. allg. Chem.*, 1916, xciv, 115. Rothmund, V., and Kornfeld, G., *Z. anorg. u. allg. Chem.*, 1918, ciii, 129. The latter article contains an extensive bibliography.

<sup>3</sup> Folin, O., and Bell, R. D., *J. Biol. Chem.*, 1917, xxix, 329.

*The Kinds of Substances Removed from Solution by Permutit.*

For the purpose of these tests about 5 cc. of the finely granular permutit prepared for urinary ammonia determinations<sup>4</sup> were poured into a calcium chloride tube, the small end of which had been loosely plugged with a very small plectet of cotton-wool or glass-wool and inserted in a 1-hole rubber stopper fitting a suction flask. The permutit was then rinsed with a few cubic centimeters of 2 per cent acetic acid to neutralize the trace of alkali usually present, then rinsed several times with distilled water, the rinsings being conveniently sucked into a flask by a filter pump. A small portion of the substance to be investigated was dissolved in 50 cc. of water. If the substance was not itself a neutral salt (usually the hydrochloride) the solution was made neutral to litmus paper by means of 0.01 N HCl or NaOH. About 40 cc. of this solution, filtered if necessary, were poured into the tube containing permutit, a test-tube was placed in the suction flask to collect the fluid as it dropped from the tube, and the filter pump so adjusted that about 1 drop each second fell in the test-tube. The first two 10 cc. portions were discarded, since they were somewhat diluted by the water with which the permutit was wet. An appropriate test was applied simultaneously to equal volumes of the original solution and of the fluid which had passed through permutit. This permitted a roughly quantitative comparison. The permutit was then rinsed five times with from 10 to 20 cc. of distilled water. A test for the substance in question was then applied, either directly to the permutit or to an alkaline or strong saline extract of the permutit. Almost without exception, substances which appeared in the filtrate were not found in the permutit.

The observations have been brought together for convenient reference in Table I. The fifth column of this table states whether or not the substance in question was removed by permutit. The words "all" and "none" should not be taken in a strictly quantitative sense, for in many cases the test used has permitted only a rough estimation.

As results of these tests accumulated it became increasingly clear that the substances removed from solution by permutit were relatively strong bases. With but one questionable exception, those bases whose "strength" is represented by a dissociation constant of  $5 \times 10^{-9}$  or greater were removed by permutit,<sup>5</sup> whereas

<sup>4</sup> Permutit, prepared for ammonia determinations, between 60 and 80 mesh, is put on the market by The Permutit Company, 440 Fifth Ave., New York City.

<sup>5</sup> The single exception is *p*-aminophenol, whose  $K_b$  is given by Veley (Veley, V. H., *J. Chem. Soc.*, 1908, xciii, 2131) as  $6.6 \times 10^{-9}$ , as estimated by his methyl orange method.

permutit took up none of the substances with a smaller basic dissociation constant.

Considered from the view-point of chemical structure it is noteworthy that in general alkylamines are removed by permutit while arylamines are not removed. The amides, including urea and its derivatives, the  $\alpha$ -amino-acids and even sarcosine, creatinine, and creatine escape capture. Of the strongly basic amino-acids, those tested by the writer (lysine and histidine) were removed from solution by permutit, and Professor Folin has also informed me that arginine is partially removed.

It may be of interest to the histologist to note that methylene blue (the commonly used nuclear stain) is taken up by permutit, and that eosin, acid fuchsin, and picric acid (the "diffuse" protoplasmic stains) are not taken up.

#### *Factors Influencing the Base-Trading Reaction of Permutit.*

The reasons for studying these factors are sufficiently obvious to require but little comment. Primarily, of course, one wishes to define the conditions under which quantitative use can be made of the reaction. A knowledge of these matters may also assist the organic chemist in separating and purifying compounds. There has also been for the writer a considerable theoretical interest because of the analogy that may exist between the base-trading by permutit and some of the most intimate chemical reactions of the living organism.

A. *Solvents.*—Permutit takes up amines not only from aqueous solutions but also from solutions in organic solvents. Quantities of tyramine and adrenalin of the order of 0.1 mg., for example are quantitatively removed from solution in 50 per cent alcohol or 98 per cent alcohol by 5 cc. of permutit which has been "dried" by five extractions with 95 per cent alcohol, followed by five extractions with absolute alcohol. The reaction is sufficiently rapid to permit the usual procedure of sucking through permutit at the rate of a drop each second. Similar results were obtained with solutions of small quantities of tyramine in amyl alcohol or in ether, using permutit "dried" as described and rinsed with amyl alcohol or ether. It should be emphasized, however, that from ether solutions permutit takes up bases which it will not take up from aqueous solutions of their salts; for example, methyl-

## "Permutit" as a Reagent for Amines

TABLE I.

Substance.	Basic dissociation constant.	Quantity. mg.	Test used.	Removed by permittit.
Ammonium chloride.....	2 $\times 10^{-5}$	1	Nessler's; reddish yellow color.	All.
Ethylamine HCl.....	5.6 $\times 10^{-4}$	5	Distillation, back titration.	"
Benzylamine HCl.....	2 $\times 10^{-6}$	10	Nessler's; white precipitate.	"
Histamine phosphate.....		5 to 10	Nessler's; precipitate.	"
Tyramine.....	"	1 to 5	Phenol reagent; blue.*	"
Hordenine sulfate.....		1	" " "	"
Adrenalin HCl.....		1	" " "	"
Choline chloride.....		1	Boiling NaOH solution; fishy odor.	"
Trimethylamine HCl.....	5.9 $\times 10^{-6}$	5 to 10	" " "	"
		1 to 5	" " "	"
Aniline HCl.....	3.1 $\times 10^{-10}$	1	Phenol reagent; blue.	None.
Toluidine HCl.....		5	" " "	"
p-Aminophenol HCl.....	6.6 $\times 10^{-9}$	1 to 5	" " "	"
Methyl-p-aminophenol sulfate.....		1 to 5	" " "	"
Diphenylamine HCl.....		1	" " "	"
m-Phenylenediamine HCl.....		1	" " "	"
Methylene blue.....		1	Its own color.	All.
Hydrazine sulfate.....	3 $\times 10^{-6}$	1	Phenol reagent; blue.	"
Phenylhydrazine HCl.....	1.62 $\times 10^{-9}$	1 to 3	" " "	None.
Semicarbazide HCl.....		1	" " "	"

Urea.....	$1.5 \times 10^{-14}$	1 to 5 1 to 5 5 to 10	Hydrolysis, Nessler's; yellow. " " " " "	None. " " " "
Acetamide.....				
Methylurea.....				
Glycine†.....	$2.8 \times 10^{-12}$	1 to 5 1 to 5 1 to 5	" " " " " " " " "	" " " " " "
Sarcosine.....	$3.1 \times 10^{-12}$	1 to 5	" " "	" "
Alanine†.....				
Leucine†.....				
Tyrosine.....				Trace.
Glutamic acid HCl.....				None.
Cysteine HCl.....				" "
Cystine.....				" "
Lysine.....				All.
Histidine 2 HCl.....	$\geq 1$ $6.7 \times 10^{-9}$	$\times 10^{-7}$ $10^{-9}$	20 5	Phosphotungstic acid; precipitate. Diaz reaction.
Piperidine.....	$1.2 \times 10^{-3}$	3	Nessler's; white precipitate. " "	" "
Pyridine.....	$1.06 \times 10^{-6}$	20	$H_2SO_4$ ; yellow precipitate.	None.
Nicotine.....		10	Phosphotungstic acid; yellow cloud.	All.
Indole.....		1	Nitrite, $H_2SO_4$ ; lavender.	None.
8-Hydroxyquinoline.....		2	Phenol reagent; blue.	About $\frac{2}{3}$ .

\* The terms "Phenol reagent" and "Uric acid reagent" refer to the phosphomolybdic and phosphotungstic acids, prepared and used according to Folin (Folin, O., Laboratory manual of biological chemistry, New York and London, 2nd edition, 1920, pp. 206-207).

† These amino-acids were contaminated by a trace of ammonia. They were purified by precipitation from alcohol with hydrochloric acid.

TABLE I—Continued.

Substance.	Basic dissociation constant.	Quantity. mg.	Test used.	Removed by permutit.
Glucosamine HCl.....	1.1 $\times 10^{-8}$	1 to 5	Copper reduction.	All.
Guanidine HCl.....	7.4 $\times 10^{-9}$	5	Nessler's; yellow precipitate.	"
Hydroxylamine HCl.....		1 to 5	Phenol reagent; blue.	"
Creatine.....		1 to 5	Picric acid, heat, NaOH; orange.	None.
Creatinine zinc chloride.....	3.69 $\times 10^{-11}$	1 to 5	" " NaOH; orange.	"
Uric acid.....		1 to 5	Phenol reagent; blue.	"
Atropine.....	>1 $\times 10^{-7}$	5 to 10	Nitrite, H <sub>2</sub> SO <sub>4</sub> , alcoholic KOH; purple.	All.
Pilocarpin.....	>1 $\times 10^{-7}$	2 to 5	Nessler's; white precipitate.	"
Congo red.....		1 to 5	Its own color.	None.
Picric acid.....		1	" color in alkaline solution.	"
Methyl red.....		1	" own color.	"
Alizarin.....		1	" color in alkaline solution.	Trace.
Eosin.....		1	" own color.	None.
Acid fuchsin.....		1	" "	"
Lecitin (in 95 per cent alcohol solution).....		5	Digestion, Bell-Doisy phosphate method.†	All.
Oleic acid (in 95 per cent alcohol solution).....		5	Turbidity with dilute HCl.	None.

† In this case only the filtrate was tested, for permuntit itself (as well as sodium silicate which has been freed of phosphate) forms with molybdic acid a substance, presumably silicomolybdc acid, which gives a blue color with alkaline hydroquinone solution. (See Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, **xiv**, 55.)

*p*-aminophenol and phenylhydrazine. Non-basic substances, however, such as picric acid, resorcinol, and pyrocatechol, are not removed from ether solutions.

For practical use with solutions in alcohol there is no need to dry the permutit in the manner described. Half an hour's gentle agitation with 5 cc. of the alcohol-dried permutit removed 78 per cent of the ethylamine from 5 cc. of 98 per cent alcohol containing 14 mg. of ethylamine N. Nearly as much (71 per cent to be exact) was removed in a parallel experiment with permutit fresh from the market, rinsed only once with absolute alcohol.

*B. Distribution of Base between Permutit and Solution.*—As has been stated above, permutit acts not by *adding* bases, but by *trading* bases. This trading is a reversible reaction and is subject to the law of mass action as will be shown below. The hydrogen ion concentration has considerable effect upon these reactions. Temperature has a perceptible influence on the rate of reaction, but not on the equilibrium.

For an approximate quantitative study of these relations methylene blue solutions are very convenient. But methylene blue is a substance which exists in two tautomeric forms.<sup>6</sup> This is probably the reason why alcohol and alkalies affect the reaction of methylene blue with permutit in a different way than they affect the reactions of other bases not having this sort of tautomerism. But with these exceptions, any one wishing to observe the behavior of permutit under varying conditions will find that experiments with methylene blue will give obvious and striking results.

To avoid interpretations and explanations based on tautomeric changes, the data presented below are taken from experiments with ammonia, which is the simplest of the nitrogenous bases and the one from which all can be considered as derived.

#### *The System—Ammonium Chloride and Sodium Permutit in Water.*

Portions of fresh permutit weighing 3 gm. were washed in a calcium chloride tube, once with 2 per cent acetic acid and three times with about 25 cc. of distilled water, and then rinsed from the inverted calcium chloride tube by a fine stream from a wash bottle into a large test-tube (25 X 200

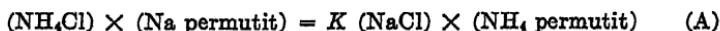
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<sup>6</sup> Kehrmann, F., and Schaposchnikoff, W., *Ber. chem. Ges.*, 1897, xxx, 1565.

mm.), calibrated at 25 cc. Into each tube was pipetted a known quantity of an ammonium chloride solution, exactly neutralized with NaOH and in some instances also a known quantity of a neutral 1.88 N NaCl solution. Distilled water was added to the 25 cc. mark. The tubes were tightly stoppered, clamped horizontally in an agitating machine which kept them in constant gentle motion for at least 12 hours. The precaution was taken to approach the equilibrium from both directions. It was thus demonstrated that 12 hours sufficed to reach a *true* chemical equilibrium. After agitation the tubes were set in racks and when the turbidity had cleared, which sometimes took an hour, the ammonia content of the supernatant fluid was determined by the colorimetric method.

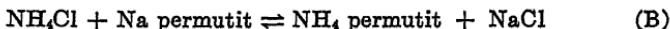
The experimental results are given in Table II where quantities are expressed in terms of hydrogen equivalents. In this table are also given the results secured in one experiment in which the volume was 50 cc. instead of 25, and another experiment in which the weight of permutit was 6 gm. instead of 3. Since there seems to be no room to doubt that in taking up ammonium the permutit releases an equivalent amount of sodium, the figures for sodium chloride at the end of the experiment have been calculated on this basis.<sup>2</sup> Sodium permutit is considered to have an equivalent weight 196 (mol. wt. 392.8).

The results indicate that the base-trading reaction of permutit is approximately in accordance with the mass law. Hissink,<sup>7</sup> who studied the liberation of ammonia from ammonium permutit when shaken in water, got results agreeing with Freundlich's empirical formula for adsorption. But the addition of sodium chloride, and even of water, plays havoc with this formula, while the relationship



remains undisturbed.

This will be recognized as the condition of equilibrium under the mass law of the reversible reaction,



It is possible from equation (A) to develop a very serviceable rule for predicting the amount of ammonia which will remain in solution at equilibrium. If the quantity of ammonia present in

<sup>7</sup> Hissink, D. J., *Landw. Vers. Sta.*, 1913, lxxxi, 377.

the system is small compared to the quantity of permutit, then at equilibrium,  $(\text{NH}_4\text{Cl})$  will be small compared to  $(\text{NaCl})$  and, since  $K$  has a value (0.75) not far from unity,  $K (\text{NaCl} + (\text{NH}_4\text{Cl}))$

TABLE II.

Experiment No.	Time.	Volume. cc.	Na permutit. $H \text{ Eq.} \times 10^4$	NH <sub>4</sub> permutit. $H \text{ Eq.} \times 10^4$	NaCl		$\frac{\text{NH}_4\text{Cl} \times \text{Na}}{\text{NaCl} \times \text{permutit.}}$ $H \text{ Eq.} \times 10^4$	$\frac{\text{NaCl} \times \text{NH}_4}{\text{NaCl} \times \text{permutit.}}$ $H \text{ Eq.} \times 10^4$
					$H \text{ Eq.} \times 10^4$	$H \text{ Eq.} \times 10^4$		
1	Beginning.	25	153	3.28	3.28	3.33	7.5	10.
	End.		149.7			0.05		
2	Beginning.	25	153	6.44	6.44	6.66	32.2	41.
	End.		146.6			0.22		
3	Beginning.	25	153	12.45	12.45	13.32	122	154
	End		140.5			0.87		
4	Beginning.	25	149.68	13.32	13.32	0.85	120	155
	End.		140.5					
5	Beginning.	50	153	12.41	12.41	13.32	128	154
	End.		140.6			0.91		
6	Beginning.	25	306	12.87	12.87	13.32	132	165
	End.		293.1			0.45		
7	Beginning.	25	153	23.14	23.14	26.64	454	533
	End.		129.9			3.50		
8	Beginning.	25	153	9.31	49.81	11.62	332	459
	End.		143.7			2.31		
9	Beginning.	25	153	8.12	88.12	11.62	507	715
	End.		144.9			3.50		
10	Beginning.	25	153	2.69	22.69	2.93	361	610
	End.		150.3			0.24		

will be very nearly the same as  $K (\text{NaCl} + \text{NH}_4\text{Cl})$ . Hence, approximately

$$\frac{(\text{NH}_4\text{ Cl})}{(\text{NH}_4\text{ permutit})} = 75 \text{ per cent } \frac{(\text{Total Cl})}{(\text{Total permutit})} \quad (\text{C})$$

That is, the ratio in which ammonia is distributed between fluid and permutit at equilibrium is about 75 per cent of the ratio of total chloride to total permutit, expressed as hydrogen equivalents.

*Reaction Rate and Temperature.*

To secure data bearing directly upon the reaction rate between ammonium chloride and sodium permutit and to determine the effect of temperature upon it, two large test-tubes were used as in the equilibrium experiments described, each tube containing 3 gm. of permutit and water to make 21 cc. The tubes were fitted inside 500 cc. bottles by means of large 1-hole cork stoppers. One bottle was filled with water at room temperature (17°C.), the other with a mixture of water and finely chopped ice, and both were allowed to stand 1 hour with the test-tubes in place and stop-

TABLE III.

*Rate of Decrease of Ammonia Concentration in Fluid Shaken with Permutit.*

Time in minutes...	Beginning	2	5	10	20	40	60	Calculated equilibrium.
0°C.	0.74	0.170	0.123	0.106	0.083	0.068	0.067	0.045
17°C.	0.74	0.181	0.102	0.082	0.073	0.068	0.067	0.045

The ammonia concentration is expressed as mg. of N per cc.

pered, to let the systems come to the desired temperatures. Then 4 cc. of a neutral ammonium chloride solution (18.6 mg. of N) were added to each tube, the tubes tightly stoppered, and each bottle was wrapped in a towel. Mixing was accomplished by gently inverting each bottle by hand, simultaneously, at the rate of about twenty-five inversions per minute. At the end of stated periods (2, 5, 10, 20, 40, and 60 minutes) the bottles were let stand a few seconds for the permutit to settle and 2 cc. removed from each tube by means of Ostwald pipettes. The ammonia content of each 2 cc. portion was subsequently determined colorimetrically, and the concentration at the moment calculated. The results are given in Table III.

At the end of the hour the ammonia distribution was practically the same in each tube and nearly the same as it should have been at equilibrium, according to the rule derived above from prolonged experiments under otherwise similar conditions. Indeed 88 per cent of the distance from initial condition to equilibrium had been covered in 2 minutes at 17°C., and (by interpolation) in 4.5 minutes at 0°C.

The influence of the temperature can best be expressed as a temperature "coefficient," calculated from the relation,

$$k_{t_2} = k_{t_1} Q_{10}^{\frac{(t_2 - t_1)}{10}} \text{ or } \log Q_{10} = \frac{\log k_{t_2} - \log k_{t_1}}{t_2 - t_1} \times 10$$

where  $k_{t_1}$  and  $k_{t_2}$  represent the reciprocals of the time required for a given change (in this case 0.22 and 0.5) and  $t_1$  and  $t_2$  represent the respective temperature (in this case 0°C. and 17°C.). The temperature coefficient so calculated is 1.6. That is, in the range between room temperature and freezing, the rate of reaction between ammonium chloride and sodium permutit is multiplied by 1.6 for each rise of 10°C.

#### *The Hydrogen Ion Concentration.*

The ability of permutit to remove bases from solution is distinctly depressed by the presence of small quantities of acids, as well as alkalies. Whereas in neutral solution 3 gm. of permutit took up 90 per cent of 26.2 mg. of ammonia N, only 83 per cent was removed in similar experiments, where 1 cc. of  $\frac{2}{3}$  N H<sub>2</sub>SO<sub>4</sub> was added, and 86 per cent when 1 cc. of  $\frac{2}{3}$  N NaOH was added. Like results were secured with ethylamine hydrochloride. The adrenalin-permutit reaction is particularly sensitive to acids. The difference in hydrogen ion concentration from a pH of 6.9 to 6.5 may cause an error as large as 20 per cent in the removal of adrenalin from solution.

#### *Advantages of Filtration over Agitation.*

Since in early experiments with adrenalin considerable quantities were destroyed when shaken with permutit, the writer was led to try the procedure of pouring the solution of adrenalin through a tube containing permutit, as described in an earlier section of this paper, in the hope that the oxidation, which was so much accelerated even by gentle agitation, might be kept within moderate limits. This manœuvre proved successful, particularly in 50 per cent alcohol at 0°C.

It was also observed that less adrenalin was removed in experiments where a solution of the hydrochloride was passed through the same permutit *two or more times*, than in experiments where an equal quantity of the solution was passed through an equal quantity of permutit but *once*. The same phenomenon can be demonstrated much more quickly and easily, with a dilute solution of methylene blue.

This point is also well exemplified by two results with ammonium chloride. When 5 cc. of a solution of ammonium chloride (containing 26.2 mg. of N) were *agitated* with 3 gm. of permutit for half an hour 2.7 mg. of N, or 10.3 per cent, were left in solution; when the same quantity was *filtered* through 3 gm. of permutit the filtrate and rinsings contained only faint traces of ammonia, varying from 0 to about 0.02 mg. of N in different experiments. Equally complete removal was obtained by filtration when the ammonium chloride solution had been diluted to 50 or 100 cc. before filtration.

Since permutit has come into use for the removal of ammonia from urine as a preliminary to urea determinations<sup>8</sup> and amino-acid determinations,<sup>9</sup> and since others may wish to make further applications, it may be well to summarize here the advantages of filtration over agitation.

1. More of the base can be removed by a given quantity of permutit, since the sodium salt formed by the reaction, which would tend to reverse it, does not accumulate.
2. Successive portions of a relatively large volume of fluid can be rapidly and expeditiously brought into intimate relation with the permutit.
3. Easily oxidizable substances are saved from destruction.

#### *Liberation of Bases Taken Up by Permutit.*

From the practical standpoint it is as important, in some cases, to recover a base from permutit as it is to have the permutit remove it from solution. In analytical work, special procedures are applicable to special cases. Thus, in the determination of urinary ammonia, the ammonia can be conveniently liberated from

<sup>8</sup> Youngburg, G. E., *J. Biol. Chem.*, 1920-21, xlv, 391.

<sup>9</sup> Folin, O., *J. Biol. Chem.*, 1922, li, 393.

permutit by sodium hydroxide solution and immediately nesslerized. And the writer has found it convenient in determinations of adrenalin to add the color reagent (Folin's uric acid reagent) directly to the permutit, then add an alkaline solution of sodium cyanide which liberates the adrenalin and develops the color at the same time.

But, when one wishes really to recover a base, or to liberate a number of bases, some of which are volatile and others not, the most generally useful reagent, in the writer's experience, has been a saturated aqueous solution of potassium chloride. It has the advantages over alkalies that it does not drive off volatile amines, nor hasten the oxidation of phenolic amines. The salt solution with which one has "extracted" the amines taken up by permutit can be freed of the great bulk of potassium chloride (and of the sodium chloride formed by its reaction with sodium permutit) by adding 3 or 4 volumes of 95 per cent alcohol, and filtering off the precipitate.

The extraction of 3 gm. of permutit containing 26.2 mg. of ammonia N with two successive 10 cc. portions of saturated KCl solution for 5 minutes each, gave 24 mg. of N, or 92 per cent. The same procedure liberated from 3 gm. of permutit 99 per cent of 10.5 mg. of ethylamine N, and even 85 per cent of 7.1 mg. of choline N.

#### *Application of Permutit to Adrenalin Determinations.*

The principles brought out in the preceding pages have enabled the writer to develop a procedure for the colorimetric estimation of adrenalin, based on its separation from uric acid and non-basic polyphenols by means of permutit, the reduction of alkaline phosphotungstate solution, and the use of a polarimeter tube in colorimetric comparison, as used by Folin and Denis for blood ammonia determinations.<sup>10</sup> Known quantities as small as 0.006 mg. of adrenalin in 50 cc. can be estimated with an accuracy of 90 per cent. And the method has given results on extracts of adrenal glands which have corresponded with physiological assay by the intestinal strip method carried out by Dr. C. J. Campbell.

Yet there are difficulties still to be overcome before the procedure can be considered a wholly satisfactory method of adrenalin

<sup>10</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.

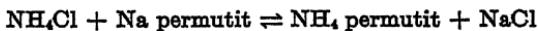
determination for biological fluids in general. Human venous blood, for example, has at times shown disconcertingly high values (*e.g.* 1.5 mg. per liter) apparently without emotional disturbance. And the high values have persisted in such blood specimens even after several days at room temperature, a phenomenon which the writer has not been able to duplicate with adrenalin in like dilutions in blood. Detailed statements of the work done and in progress are reserved for later publication.

The writer wishes to express his thanks to Professor Folin for encouragement in this work and for advice in arranging it for publication.

#### SUMMARY AND CONCLUSIONS.

1. The synthetic zeolite "permutit" can be used to separate relatively strong nitrogen bases (those having a basic dissociation constant of about  $5 \times 10^{-9}$  or greater) from weaker nitrogen bases and non-basic substances.

2. The system ammonium chloride and sodium permutit in water obeys the mass law according to the reversible reaction,



In this system the equilibrium constant  $K$  has a value 0.75 in the expression

$$(\text{NH}_4\text{Cl}) (\text{Na permutit}) = K (\text{NH}_4 \text{ permutit}) (\text{NaCl})$$

The temperature coefficient  $Q_{10}$ , in the range between room temperature and 0°C., has a value 1.6 in the expression

$$k_{t_2} = k_{t_1} \frac{(t_2 - t_1)}{10} Q_{10}$$

3. In half hour tests more of a given base is removed from a neutral solution than from an acid or alkaline solution.

4. Permutit removes bases from solution in varying concentrations of ethyl alcohol, in amyl alcohol, and in ether.

5. For the quantitative removal of bases from solution, filtration through permutit has several advantages over agitation with permutit.

6. A saturated solution of potassium chloride is a good general reagent for the recovery of bases taken up by permutit. Special procedures can be used in special cases.

7. A procedure for adrenalin determinations can be based upon its separation by permutit from many interfering substances.

# STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN THE BLOOD.

## V. FACTORS CONTROLLING THE ELECTROLYTE AND WATER DISTRIBUTION IN THE BLOOD.\*

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\* The results given in this paper were presented at the meeting of December 4, 1922, to the Peking section of the Society for Experimental Biology and Medicine, and an abstract is published in the Proceedings for that meeting.

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#### THEORETICAL.

##### INTRODUCTION.

The present paper is devoted to the quantitative formulation and experimental trial of a physicochemical explanation for the distribution of electrolytes and water between cells and plasma, and of the influence on the distribution of such factors as addition or loss of carbon dioxide, oxygen, acids, alkali, and salt. Such an explanation would cover, among other things, the facts that chloride and bicarbonate anions, though freely diffusible through the cell membrane, may be only half as concentrated in the cells as in the serum, and that the cell contents are more acid than the serum, although the  $H^+$  (or  $OH^-$ ) ion also is freely diffusible. It would cover also the mechanisms by which the cell buffers, though not diffusible, exert their buffer effects in the serum, and by which the cells take water from the serum when the carbon dioxide tension is raised. It is furthermore probable that the same laws which govern water and electrolyte distribution between intracellular and extracellular fluids in the blood, also govern these distributions in the other parts of the body, and control the relationships between the tissue cells and extracellular fluids, such as lymph and transudates.

The information concerning the amounts of base bound by oxyhemoglobin and reduced hemoglobin published in the third (1) and fourth (2) papers of this series indicated the possibility of a theoretical solution of the problem based upon the physicochemical laws of solutions, and upon the known facts concerning the reaction of the blood and the nature of its diffusible and non-diffusible ions and molecules.

*Résumé of Facts Concerning the Blood Electrolytes.*

The facts concerning the blood which may be accepted on the basis of data already in the literature are the following:

1. The osmotic pressure of the fluid within the cells appears to equal that of the serum outside. The disc shape of the erythrocyte indicates the absence of internal pressure. The latter would force the cell to assume a globoid shape, as it tends to do in hypotonic solutions.

2. In both cells and serum the positive charges of the alkali cations are balanced in part by negatively charged, non-diffusible protein anions, and in part by diffusible anions, of which  $\text{Cl}'$  and  $\text{HCO}_3'$  constitute the greater part.

3. All the non-protein ions normally present in amounts contributing significantly to the total osmotic pressure are monovalent. These are  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Cl}'$ , and  $\text{HCO}_3'$ .  $\text{Mg}^{++}$ ,  $\text{Ca}^{++}$ ,  $\text{SO}_4''$ , and  $\text{HPO}_4'''$  are present in relatively such small amounts that in an approximation of conditions controlling the total osmotic pressure they may be neglected.

In Kramer and Tisdall's (3) summary of the results of analyses of the inorganic constituents of human serum, out of a total of 0.2912 gram equivalent of basic and acid radicles per liter of serum, Na, K, Cl, and  $\text{HCO}_3$  accounted for 0.2787, or 95.6 per cent of the total. Of the remainder Ca accounted for 0.0050 equivalent, or 1.8 per cent of the total, Mg for 0.9 per cent,  $\text{HPO}_4$  for 0.0010 equivalent, or 0.3 per cent, and  $\text{SO}_4$  for 0.004 equivalent, or 1.4 per cent.

In the cells Kramer and Tisdall estimate amounts of K,  $\text{HCO}_3$ , and Cl accounting for 0.2001 out of a total of 0.2061 equivalent, or for 97 per cent of the total.

The cell inorganic phosphates on the basis of older analyses were, by one of us in a previous review (4) and in the introduction to the first paper of this series (5), classified with the non-diffusible electrolytes and accredited with an important share in the total osmotic and buffer effect. Using methods designed to avoid decomposing organic phosphates, Zucker and Gutman (6) have recently found that the blood phosphates are present in such small amounts as to play a quantitatively insignificant part in the total buffer and osmotic effects of the blood, and that they are probably diffusible.

4. Of the cell and serum proteins, only hemoglobin exerts a significant part of the total osmotic pressure.

That the electrolyte molecules and ions constitute nearly all of the osmotically active substances present is shown by the correspondence between the lowering of the vapor tension observed (Neuhausen, 7) and the lowering attributable to the electrolytes present. The chief non-electrolyte crystalloids, glucose and urea, themselves diffuse through the cell membranes and therefore cannot influence the water distribution. They are furthermore present in relatively small amounts, about 5 and 3 millimols respectively out of a total osmolar concentration of 300.

Of the proteins it appears that hemoglobin is the only one that exerts more than 1 per cent of the total osmotic pressure in either cells or serum. Hüpner and Gansser (8) found that electrolyte-free ox and horse hemoglobin exert the osmotic pressures calculated on the assumption that 1 molecule of oxygen combines at atmospheric pressure with 1 molecule of hemoglobin, and we have based our calculations of the osmotic effect of hemoglobin on these results. It will be seen from the tables that hemoglobin is estimated to exert about 10 per cent of the total osmolar concentration of the cells.<sup>1</sup>

The serum proteins, according to Starling (9) exert 30 to 40 mm. of pressure, or less than 1 per cent of the total (estimated at  $0.300 \times \frac{311}{273} \times 22.4 \times 760 = 5,800$  mm.). Presumably the cell proteins other than hemoglobin exert still less pressure, because of their small amount. It appears therefore that in calculating the total osmotic effects of the blood, the serum proteins and the cell proteins other than hemoglobin may be neglected.

We have adopted the convenient term "osmolar" concentration introduced by Warburg (10), to indicate the total concentration of osmotically active ions and molecules.

##### 5. The cell membranes are permeable to water, carbon dioxide, to the inorganic anions, and to either $H^+$ or $OH^-$ , or both.

In water solutions the same  $[H^+]$  would result, whether the membrane is permeable to  $[H^+]$ , or  $[OH^-]$ , or to both. The concentration of either ion varies inversely as that of the other, according to the equation  $[H^+] = \frac{K_w}{[OH^-]}$ . Any factor which fixes  $[H^+]$  therefore fixes  $[OH^-]$ , and *vice versa*, so that it is impossible, and likewise for our present purposes immaterial, to tell whether the membrane is permeable for  $[H^+]$ ,  $[OH^-]$ , or both.

<sup>1</sup>Adair, in a personal communication, states that measurements that he has made in L. J. Henderson's laboratory indicate in dilute solutions a much higher molecular weight for hemoglobin than that found by Hüpner and Gansser, but that in concentrations approaching those in the cells other forces augment the osmotic power of the hemoglobin to about that which corresponds to Hüpner and Gansser's measurements.

6. The cell membranes are impermeable to the proteins, ionized or not, and to K and Na (Gürber, 11; Doisy and Eaton, 12).

7. The physiological pH ranges of the cells and serum are on the alkaline side of the isoelectric points of the cell and serum proteins (Michaelis, 13). Consequently, in the body the blood proteins combine with alkalies, but not with acids in amounts significant for the purposes of this paper.

8. The amounts of alkali bound by the cell and serum proteins increase in approximately a linear manner with increasing pH over the physiological range. The rate of change in protein-bound alkali per unit change in pH is several times as great in the cell fluid as in the serum (2).

9. At physiological pH ranges reduced hemoglobin binds less alkali (0.5 to 0.7 equivalent less per molecule of hemoglobin) than does oxygenated hemoglobin (1, 2).

#### *The Solution Laws Involved in Blood Relationships.*

In combining the above facts to form an inclusive quantitative expression of the phenomena of electrolyte and water distribution we have assumed for the blood the validity of the following physicochemical laws:

I. At and near the neutral point all strong alkalies in quantitatively significant amounts are in the form of salts. At blood reaction, therefore, the total base B may be represented as BP + BA, where BP represents the alkali protein salts, in equivalents of monovalent alkali, and BA the salts formed by the alkali with other negative radicles, chiefly Cl' and HCO<sub>3</sub>'.

II. The law of Donnan governing the influence of non-permeating ions on the distribution of permeating ions on the two sides of a membrane holds for the membranes of the blood cells. Donnan's theory has been provided with a basis of experimental facts by Donnan (14), by Procter and Wilson (15), and, for protein solutions, especially by Loeb's (16) recent studies.

III. The osmotic activity of each solute is proportional to the ratio  $\frac{n}{N}$  of gram molecules of solute to gram molecules of water.

The presence of the serum proteins, according to the vapor tension determinations of Neuhausen (7), does not affect the validity of

this ratio as the governing factor of osmotic activity, and data to be presented in this paper show that the cell proteins likewise fail to affect it. With dilute water solutions it makes relatively little difference whether the ratio  $\frac{n}{H_2O}$  or  $\frac{n}{\text{volume}}$  is taken as a measure of osmotic activity. In the blood cells, however, where the water constitutes only 60 to 65 per cent of the total contents, the difference is of importance.

Bjerrum (quoted by Warburg (10)) considers the ratio  $\frac{n}{N+n}$  to be a better indicator of osmotic activity in concentrated solutions than the ratio  $\frac{n}{N}$ . In blood, however,  $n$  is less than 0.01 as great as  $N$ , so that within the limits of experimental error it is immaterial which of these two ratios we use. We consequently shall employ the simpler,  $\frac{n}{N}$ .

For our calculations, in place of using gram molecules of water as the unit of the denominator, we have used kilos of water, in order to express the results in terms not unnecessarily removed from the familiar gram molecules per liter unit.

The relationships expressed above under I, II, and III, may be expressed in certain basic equations, which when combined yield a practicably simple expression indicating the quantitative relationships of the factors discussed.

I. For the approximate *neutrality* of the blood reaction,  $[OH^-]$  and  $[H^+]$  being negligible compared with the other ions, we have

$$(1) \quad [B]_s = [BA]_s + [BP]_s$$

$$(2) \quad [B]_c = [BA]_c + [BP]_c$$

The brackets are used to indicate concentrations in terms of the ratio  $\frac{\text{solute}}{\text{water}}$ . The subscripts  $s$  and  $c$  indicate serum and cells, respectively. B, BA, and BP have the significance used under I in the preceding discussion. (For simplicity we indicate all the alkali bound to non-diffusible acids as BP, although a small part may be bound by substances other than proteins, such as conjugated phosphates.)

II. For conformity with *Donnan's law* (14) the *diffusible* monovalent ions have the following relationships:<sup>2</sup>

$$(3) \quad \frac{[H^+]_s}{[H^+]_c} = \frac{[Cl']_c}{[Cl']_s} = \frac{[HCO_3']_c}{[HCO_3']_s} = \frac{[OH']_c}{[OH']_s} = \frac{[A']_c}{[A']_s} = r .$$

$A'_c$  and  $A'_s$  represent the sums of all the monovalent anions. For convenience we shall use the factor  $r$  to express the ratio indicated.

$[B]_c$  and  $[B]_s$  do not appear in Equation 3, for they are not diffusible. If they were, in addition to the conditions defined in Equations 1, 2, 3, and 4, we should be required to make our final equation conform to the condition that  $\frac{[Na]_s}{[Na]_c} = \frac{[K]_s}{[K]_c} = r$ , and the results would be altogether different.

The relationships expressed in Equation 3 have already been pointed out by Warburg (10) and by Barcroft, Bock, Hill, Parsons, Parsons, and Shoji (17).

Barcroft and his collaborators (17), however, apparently used as a measure of activity the  $\frac{\text{molecules of solute}}{\text{volume of solute}}$  ratio which is valid for dilute solutions of substances of small molecular weight. Warburg in his consideration of the problem realized that the volume ratio is not a close measure of activity in solutions containing as great a bulk of protein as the cell contents, and multiplied by the factor  $\frac{\alpha_{CO_2} \text{ in water}}{\alpha_{CO_2} \text{ in blood}}$  the  $H_2CO_2$  concentration per unit volume, but not the concentrations of the other solutes.

In applying Donnan's law Barcroft and his collaborators have assumed that the amount of alkali bound by hemoglobin is negligible. "Of the total ionic charges inside the corpuscles hemoglobin can provide, owing to its enormous molecular weight, only a negligible part." Since the appearance of Barcroft and his collaborators' paper, the experiments of Van Slyke, Hastings, Heidelberger, and Neill (1) have demonstrated that because of the polyvalent acid character of the hemoglobin molecule it combines at pH 7.4 with an amount of alkali in the cell about equal to that in the form of chloride. Of equal importance is the fact that practically all the changes in the ionic content of the cell that occur with vary-

<sup>2</sup> For a divalent anion, such as  $SO_4^{''}$  or  $HPO_4^{''}$ , the distribution factor, according to Donnan's law is indicated by

$$r = \frac{[Cl']_c}{[Cl']_s} = \frac{\sqrt{[SO_4^{''}]_c}}{\sqrt{[SO_4^{''}]_s}} = \frac{\sqrt{[HPO_4^{''}]_c}}{\sqrt{[HPO_4^{''}]_s}}$$

ing  $\text{CO}_2$  and  $\text{O}_2$  tensions originate in the variations which  $\text{CO}_2$  and  $\text{O}_2$  tensions cause in the amounts of alkali bound by hemoglobin.

Warburg developed formulas in which the buffer effects of (oxygenated) cell and plasma proteins were indicated, but in which the actual amounts of base balanced by negative protein charges were not introduced. The data for estimating these amounts were, of course, not available at the time of Warburg's publication.

III. For *osmotic equality* the ratio of osmotically active molecules and ions to water is the same in serum and cells.

$$(4) \quad \frac{\Sigma M_s}{\text{H}_2\text{O}_s} = \frac{\Sigma M_c}{\text{H}_2\text{O}_c} \text{ or } \Sigma [M]_s = \Sigma [M]_c$$

In Equation 4,  $M_s$  and  $M_c$  represent the osmotically active ions and molecules, and  $\Sigma [M]_s$  and  $\Sigma [M]_c$ , the sums of their total concentrations, in terms of the  $\frac{\text{solute}}{\text{water}}$  ratio, in serum and cells, respectively.

As alternative forms of Equation 4, we may write, if we assume complete dissociation of the electrolytes:

$$(5) \quad [\text{B}]_s + [\text{Cl}]_s + [\text{HCO}_3]_s = [\text{B}]_c + [\text{Cl}]_c + [\text{HCO}_3]_c + [\text{Hb}]_c$$

$$(6) \quad 2[\text{BA}]_s + [\text{BP}]_s = 2[\text{BA}]_c + [\text{BP}]_c + [\text{Hb}]_c$$

$$(7) \quad 2[\text{B}]_s - [\text{BP}]_s = 2[\text{B}]_c - [\text{BP}]_c + [\text{Hb}]_c$$

Equation 5 merely expresses the sum of the total ions in serum, and of ions plus hemoglobin molecules in the cells, complete dissociation being assumed, and likewise a balancing, in serum and cells, respectively, of the small amount (not over 5 per cent of the total) of osmotically active substances ( $\text{PO}_4^{''}$ ,  $\text{SO}_4^{''}$ , etc.) not represented in the equation. Hb is expressed in units of mols of  $\text{O}_2$  capacity.

In Equation 6 the total osmolar concentration is represented as twice the molecular concentration of the salts with monovalent ions and cations (since each dissociates into two ions) plus once the concentration of base in the form of protein salt, since the osmotic effect of BP is due to the alkali cation. In the cells we add also the osmotic effect of the hemoglobin, which is assumed to be the same regardless of the ionic charge of the hemoglobin molecules.

Equation 7 is derived from Equation 6 by substituting  $[\text{B}] - [\text{BP}]$  for  $[\text{BA}]$ , according to Equations 1 and 2.

As stated above, Equations 5, 6, and 7 are theoretically accurate if the electrolytes are completely dissociated into osmotically active ions. The observed osmotic behavior of alkali salts in general does not justify the assumption that dissociation is complete, and Neuhausen and Marshall (18) from electrometric measurements have estimated that the sodium salts in blood serum are 83 per cent dissociated. However, if we assume, not complete dissociation, but *equal* dissociation of the salts in cells and serum, respectively, the relationships expressed in Equations 5, 6, and 7 still hold, not exactly, but so nearly that the deviations may be neglected for present purposes.

The theoretical inexactness of Equations 5, 6, and 7 when  $\gamma$ , the degree of dissociation, is less than 1, even though  $\gamma$  is equal on both sides of the membrane, arises as follows. When  $\gamma$  becomes less than 1, although  $[Cl]$ ,  $[HCO_3]$ , and the part of  $[B]$  balanced by  $[Cl]$  and  $[HCO_3]$ , are all multiplied on both sides of the equation by the same factor,  $\frac{1+\gamma}{2}$ , to give their osmotic activities, the part of  $B$  present as  $BP$  is multiplied by a smaller factor,  $\gamma$ , and the  $[Hb]$  by a larger factor, 1. The two deviating factors,  $\gamma$  and 1, however, are not greatly different from  $\frac{1+\gamma}{2}$ , which is their mean; they apply in blood to relatively small parts of the total osmotically active solutes; and they partially balance their effects, which, to judge from our experimental results, exceed but little if any our present limits of experimental measurement.

The basic assumptions made under I and II, and expressed in Equations 1, 2, and 3, stand on experimental data familiar in the literature. The assumption of equal  $\frac{\text{solute}}{\text{water}}$  ratios in cells and serum, expressed under III in Equation 4, and in Equations 5, 6, and 7, is without experimental basis in the previous literature. It depends upon data in the present paper. These are summarized in Table I, taken from the four experiments with horse blood described in the experimental section of the paper. The data given are those that fit into Equation 5, because these are all determined by direct analysis. Except in Blood 2, in which we believe the base determinations on the cells were low, the agreement is as close as the analyses could be expected to yield.

*Electrolyte Distribution.*

Dividing Equation 6 through by 2 [BA]<sub>s</sub> and rearranging it we obtain

$$(8) \quad \frac{[BA]_c}{[BA]_s} = 1 - \frac{[BP]_c + [Hb]_c - [BP]_s}{2 [BA]_s}$$

We may assume that, whatever the dissociations of the different salts with the monovalent anions, the salts with identical anions are dissociated to nearly the same extent in serum and cells.

TABLE I.  
*Equality of Ratio  $\frac{\text{Mols Solute}}{\text{Water}}$  in Cells and Serum.*

Blood No.	pH <sub>s</sub>	Serum.	
		B + Cl + HCO <sub>3</sub>	Cells. B + Cl + HCO <sub>3</sub> + Hb
1	7.66	289	272
	7.11	299	294
2	7.71	297	285
	7.42	307	292
	7.28	314	297
	7.11	318	299
3	7.75	286	285
	7.42	294	294
	7.08	303	303
4	7.69	290	289
	7.35	297	294
	7.06	302	303

so long as the concentrations do not differ greatly. This assumption appears justified even though the cations in the cell are nearly all K, while those in the serum are nearly all Na; for, whether conductivity or freezing point data are considered, the differences in dissociation found between potassium and sodium salts with the same anions at similar concentrations are slight.<sup>3</sup> We may then write, with approximate accuracy

$$(9) \quad \frac{[BA]_c}{[BA]_s} = \frac{[A']_c}{[A']_s} = r$$

<sup>3</sup> See tables in Lewis (19), pp. 226-227.

(From Equation 3,  $\frac{[A']_s}{[A']_c} = r$ .) From Equation 1,  $[BA]_s = [B]_s - [BP]_s$ . Substituting, in Equation 8,  $r$  for  $\frac{[BA]_s}{[BA]_c}$  in the left hand member, and  $[B]_s - [BP]_s$  for  $[BA]_s$  in the right hand member, we obtain the following equation, showing the approximate relationship between the distribution of diffusible ions and the amounts of alkali combined with the non-diffusible substances (proteins) of the cells and serum.

$$(10) \quad r = \frac{[\text{H}^+]_s}{[\text{H}^+]_c} = \frac{[\text{Cl}]_c}{[\text{Cl}]_s} = \frac{[\text{BHCO}_3]_s}{[\text{BHCO}_3]_c} = 1 - \frac{[\text{BP}]_s + [\text{Hb}]_s - [\text{BP}]_c}{2 ([\text{B}]_s - [\text{BP}]_s)}$$

We may expect the three ratios in Equation 10 to vary from equality to each other, and to the  $r$  calculated from the right hand member of the equation, in proportion as the  $\gamma$ , and perhaps secondary factors affecting osmotic activity, vary in the cells from the like factors in the serum, but we may expect these variations in the ratios not to exceed a few per cent of their values.<sup>4</sup>

To obtain the value of  $r$  in terms indicating directly the effect of protein concentration, pH values, and degree of oxygenation, we substitute for  $[\text{BP}]_s$  and  $[\text{BP}]_c$  their values in these terms, as found in the experimental part of this paper and expressed in Equations 54 and 57. Making these substitutions in Equation 10 and combining the terms in which  $[\text{Hb}]_s$  is a factor, we obtain, as an approximation, accurate over a narrow pH range,

$$= 1 - \frac{3.35 [\text{Hb}]_s (\text{pH}_s - 6.44) - 0.068 [\text{P}]_s (\text{pH}_s - 4.80) + [\text{O}_2]_s (0.25 \text{ pH}_c - 1)}{2 ([\text{B}]_s - 0.068 [\text{P}]_s \{\text{pH}_s - 4.80\})}$$

<sup>4</sup> The relationship between electrolyte distribution and alkali bound by protein may also be derived as follows. From Equations 1 and 2,  $[\text{A}]_s = [\text{B}]_s - [\text{BP}]_s$ , and  $[\text{A}]_c = [\text{B}]_c - [\text{BP}]_c$ .

$$\text{Hence } r = \frac{[\text{A}]_s}{[\text{A}]_c} = \frac{[\text{B}]_s - [\text{BP}]_s}{[\text{B}]_c - [\text{BP}]_c}$$

This equation rests only on the assumption expressed in Equations 1, 2, and 3, and does not express or depend upon the equality of the  $\frac{\text{solute}}{\text{water}}$  ratios expressed in Equation 4. It could not therefore be used for the further development of the theory into equations like Equations 14 and 23, which depend on the validity of the  $\frac{\text{solute}}{\text{water}}$  ratio.

Equations 10 and 11 suffice for determining whether results obtained with a given blood agree with the quantitative requirements of the laws on which these equations are based. Because of the variation in water distribution with changing pH and oxygen content, however, the concentrations even of the non-diffusible constituents  $[Hb]_c$ ,  $[P]_s$ , and  $[B]_s$  are variable. Consequently, Equations 10 and 11 cannot be used to predict the  $r$  curve of a given blood with varying pH. However, by combining Equation 4 with 10, one is obtained in which all the values on the right side are functions of values which are constant for a given blood; viz.,  $(B)_c$ ,  $(B)_s$ ,  $(Hb)$ , and  $(P)_s$ .

In the remainder of this paper we shall utilize parentheses to indicate units of substance *per unit of whole blood*, e. g.  $(H_2O)_c$  = kilos of cell water per kilo of blood,  $(P)_s$  = grams of serum protein per kilo of blood, and  $(B)_s$  = millimols of serum base per kilo of blood, as contrasted with the bracketed  $[B]_s$ , which indicates the ratio  $\frac{\text{serum base}}{\text{serum water}}$ , or  $\frac{(B)_s}{(H_2O)_s}$ .

In Equation 10 we substitute  $\frac{(BP)_s}{(H_2O)_s}$  for  $[BP]_s$ ,  $\frac{(Hb)_s}{(H_2O)_s}$  for  $[Hb]_s$ , etc. We thus obtain

$$(12) \quad r = 1 - \frac{(H_2O)_s}{(H_2O)_c} \times \frac{(BP)_s + (Hb)}{2 \{ (B)_s - (BP)_s \}} + \frac{(BP)_s}{2 \{ (B)_s - (BP)_s \}}$$

From Equation 4 we have  $\frac{(H_2O)_s}{(H_2O)_c} = \frac{\Sigma M_s}{\Sigma M_c}$ . Substituting  $\frac{2 (B)_s - (BP)_s}{2 (B)_s - (BP)_s + (Hb)_s}$  for  $\frac{\Sigma M_s}{\Sigma M_c}$  (see discussion of Equation 7) we get

$$(13) \quad \frac{(H_2O)_s}{(H_2O)_c} = \frac{2 (B)_s - (BP)_s}{2 (B)_s - (BP)_s + (Hb)_s}$$

Substituting in Equation 12 the value for  $\frac{(H_2O)_s}{(H_2O)_c}$  from Equation 13, we obtain

$$(14) \quad r = \frac{[H^+]_s}{[H^+]_c} = \frac{[Cl']_c}{[Cl']_s} = \frac{[HCO_3']_c}{[HCO_3']_s} = 1 - \frac{(BP)_s + (Hb)}{2 (B)_s - (BP)_s + (Hb)} + \frac{(BP)_s}{2 \{ (B)_s - (BP)_s \}}$$

The effects of the pH and oxygen content may be introduced as in Equation 11 by substituting the values of  $(BP)_c$  and  $(BP)_s$  from Equations 54 and 57.

If the indiffusible substances, base and proteins, in the cells are assumed to maintain constant relations to each other, and the indiffusible substances within the serum are assumed to do likewise, it becomes possible to express as functions of  $(Hb)$  the other three constants,  $(P)_s$ ,  $(B)_s$ , and  $(B)_c$ . Under these circumstances  $(B)_c$  is proportional to  $(Hb)$ , and the serum base and protein,  $(B)_s$  and  $(P)_s$ , decrease by amounts proportional to  $(Hb)$ . Thus, from the data in the experiments on normal horse blood reported in this paper, we have with a fairly close degree of constancy:

$$(15) \quad (B)_c = 6.0 \text{ (Hb)}$$

$$(16) \quad (B)_s = 148 - 8.3 \text{ (Hb)}$$

$$(17) \quad (P)_s = 0.072 - 0.0039 \text{ (Hb)}$$

In Equations 16 and 17 the first numerical constant in each represents the average value at normal pH for serum free from cells, and when, therefore,  $(Hb) = 0$ . The second constants indicate the rates of change in  $(B)_s$  and  $(P)_s$ , respectively, per unit of increase in hemoglobin, when the hemoglobin is measured in terms of millimols of oxygen capacity per kilo of blood.

From inspection of Equation 14 it is evident that the fraction  $\frac{(BP)_c + (Hb)}{2(B)_s - (BP)_s + (Hb)}$ , expressing the effects of the cell factors, is at a given pH, constant for all bloods, whether of high or low hemoglobin content, as long as the ratio of base to hemoglobin in the cells remains constant. For then all the terms in both numerator and denominator vary directly as  $(Hb)$  (see Equations 15 and 55). The second fraction of Equation 14,  $\frac{(BP)_s}{2 \{(B)_s - (BP)_s\}}$ , expressing the effect of the serum factors, varies slightly, at constant pH, with the hemoglobin content of the blood. But the variation is so small, and the total effect of this fraction on the

value of  $r$  relatively so little, that the  $r$  value is, within the limits of experimental determination, independent of the hemoglobin content of the blood, even when the latter varies over such a wide range as from 3 to 12 millimolar, corresponding to from 7 to 27 cc. of oxygen capacity per 100 gm. of blood.

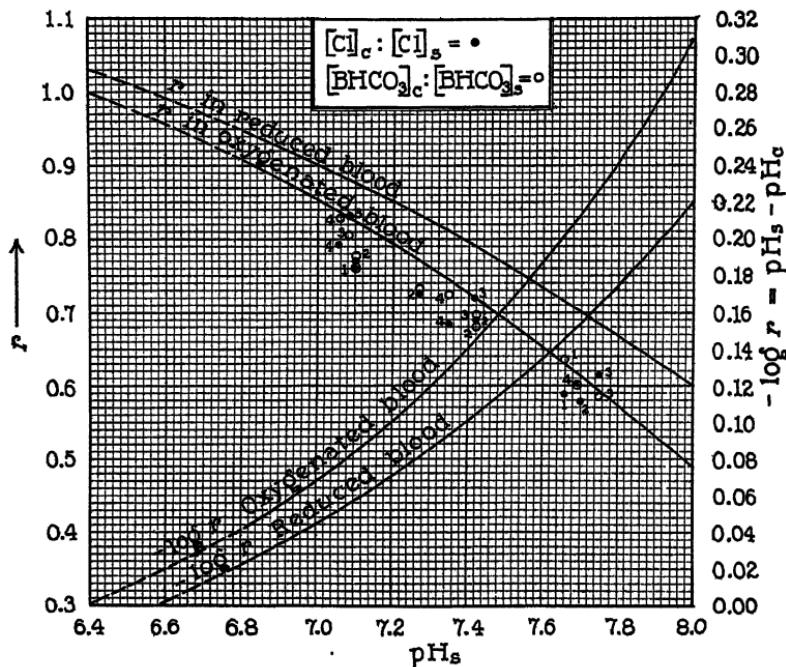


FIG. 1. Values of  $r = \frac{[H^+]_c}{[H^+]_s} = \frac{[Cl']_c}{[Cl']_s} = \frac{[HCO_3']_c}{[HCO_3']_s}$ , calculated by Equation 14 for horse blood of average serum and cell composition observed in the four experiments reported in this paper are indicated by the curves. The observed chloride and bicarbonate ratios in our experiments are indicated by the marked points.

Consequently we may represent the average normal  $r$ , pH relationship by a single curve, which holds for bloods of varying hemoglobin content, if the other non-diffusible constituents maintain towards the hemoglobin the relationships indicated by Equations 15, 16, and 17. The curves obtained by substituting in Equation 14 the values for  $(B)_c$  and  $(B)_s$  indicated by Equa-

tions 15 and 16, (when Hb = 9) and the values for (BP)<sub>s</sub>, calculated from the (P)<sub>s</sub> indicated by Equation 17, is given in Fig. 1. In plotting the curves the values of  $r$  were obtained by solving Equation 14 by a method of repeated approximation, for pH<sub>s</sub> values of 6.4, 6.8, 7.2, 7.6, and 8.0. For the first approximation the serum factors were ignored, and  $r$  was estimated as

$$1 - \frac{(BP)_s + (Hb)}{2(B)_s - (BP)_s + (Hb)}$$

(BP)<sub>s</sub> being estimated by interpolation on the curves of Fig. 10 (below pH<sub>s</sub> 6.8 the curves are extrapolated beyond experimental data, and therefore are not so certain as above that point). From the preliminary  $r$  values thus obtained, a preliminary pH<sub>s</sub> was estimated as pH<sub>s</sub> = pH<sub>e</sub> + log  $r$ . A close approximation of  $r$  was then obtained by using the whole of Equation 14, with this pH<sub>s</sub> value for estimation of (BP)<sub>s</sub>, by Equation 54 in the fraction  $\frac{(BP)_s}{2\{(B)_s - (BP)_s\}}$ . With the second value of  $r$  thus obtained the value of this fraction was once more estimated, and with it the final value of  $r$ , correct within a negligible limit of error.

In Fig. 1 the values for  $r$  obtained in the four experiments with oxygenated blood described later in this paper are indicated by solid circles for the  $\frac{[Cl]_s}{[Cl]_e}$  ratio, by hollow circles for the  $\frac{[BHCO_3]_s}{[BHCO_3]_e}$  ratio. Their deviations from the mean calculated curve are partly due to the fact that the theoretically calculated  $r$  values tend to lie above the observed values, but more to the fact that the relationships of (B)<sub>s</sub>, (B)<sub>e</sub>, and (P)<sub>s</sub> to (Hb) vary in the individual bloods appreciably from the mean relationships indicated in Equations 15, 16, and 17, and used in plotting the curves of Fig. 1. In Fig. 12, where the individual (B)<sub>s</sub>, (B)<sub>e</sub>, and (P)<sub>s</sub>, as well as (Hb), values found in each individual blood are used, the differences between the calculated and observed  $r$  values are smaller.

Since, according to Donnan's law as expressed in Equation 3,  $r = \frac{[H^+]_s}{[H^+]_e}$ , we may write

$$(18) \quad - \log r = - \log [H^+]_s + \log [H^+]_e \\ = pH_s - pH_e$$

The values of  $-\log r$ , therefore, indicate the pH differences between the serum and cells. These values we have plotted in the curves indicated in Fig. 1.

From data of quite a different nature, obtained on whole blood, serum, and hemolyzed blood and cells, and based in part on electrometric determinations, Warburg (10) has estimated the  $\text{pH}_s - \text{pH}_c$  values in horse blood at varying  $\text{pH}_s$ . Comparison shows that our  $-\log r$  curve is parallel throughout and nearly identical with the curve indicating the maximum  $\text{pH}_s - \text{pH}_c$  values estimated by Warburg.<sup>5</sup>

#### *Water Distribution and Cell Volume.*

The distribution of water between cells and serum, and the resulting volume effects, may be predicted from the pH and the degree of oxygenation of the blood if the amounts of non-diffusible substance, *viz.* base and protein, in the cells and serum, respectively, are known, and if the law of equality of osmolar concentrations expressed in Equation 4 is valid for blood, as we believe it is demonstrated to be by experimental data in this paper.

From the general statement expressed in Equation 4 we have

$$(19) \quad \frac{(\text{H}_2\text{O})_s}{(\text{H}_2\text{O})_b} = \frac{\Sigma (\text{M})_s}{\Sigma (\text{M})_b} = \frac{\Sigma (\text{M})_s}{\Sigma (\text{M})_s + \Sigma (\text{M})_c}$$

$$(20) \quad \frac{(\text{H}_2\text{O})_c}{(\text{H}_2\text{O})_b} = \frac{\Sigma (\text{M})_c}{\Sigma (\text{M})_b} = \frac{\Sigma (\text{M})_c}{\Sigma (\text{M})_s + \Sigma (\text{M})_c}$$

where  $(\text{H}_2\text{O})_s$ ,  $(\text{H}_2\text{O})_c$ , and  $(\text{H}_2\text{O})_b$ , represent the fractions of a kilo of water present, respectively, in the serum, cells, and whole of a kilo of blood,  $\Sigma (\text{M})_s$ ,  $\Sigma (\text{M})_c$ , and  $\Sigma (\text{M})_b$ , the total osmolar units (millimols) in the serum, cells, and whole of a kilo of blood.

Substituting for  $\Sigma (\text{M})_c$  and  $\Sigma (\text{M})_s$  their values as in Equation 7, and replacing  $(\text{B})_c + (\text{B})_s$  by  $(\text{B})_b$  in the resulting equations, we obtain

$$(21) \quad \frac{(\text{H}_2\text{O})_s}{(\text{H}_2\text{O})_b} = \frac{2(\text{B})_s - (\text{BP})_s}{2(\text{B})_b - (\text{BP})_s - (\text{BP})_c + (\text{Hb})}$$

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<sup>5</sup> See Warburg (10), p. 230, Curve I, Fig. 11.

$$(22) \quad \frac{(\text{H}_2\text{O})_c}{(\text{H}_2\text{O})_b} = \frac{2(\text{B})_c - (\text{BP})_c + (\text{Hb})}{2(\text{B})_b - (\text{BP})_s - (\text{BP})_c + (\text{Hb})}$$

Multiplying Equations 21 and 22 through by  $(\text{H}_2\text{O})_b$ , we obtain

$$(23) \quad (\text{H}_2\text{O})_s = (\text{H}_2\text{O})_b \times \frac{2(\text{B})_s - (\text{BP})_s}{2(\text{B})_b - (\text{BP})_s - (\text{BP})_c + (\text{Hb})}$$

$$(24) \quad (\text{H}_2\text{O})_c = (\text{H}_2\text{O})_b \times \frac{2(\text{B})_c - (\text{BP})_c + (\text{Hb})}{2(\text{B})_b - (\text{BP})_s - (\text{BP})_c + (\text{Hb})}$$

The above equations, the validity of which has been tested in the experimental part of this paper, enable one to predict the amounts of cell and serum water per unit weight of blood in terms which are either determinable constants  $(\text{B})_c$ ,  $(\text{B})_s$ , and  $(\text{Hb})$ , for a given blood, or which may be calculated from such constants, *viz.*  $(\text{P})_s$  and  $(\text{Hb})$ , and from the pH and oxygen content. The effects of pH and of oxygen saturation may be introduced as in Equation 11.

Within limits the increase of volume produced by adding a solute to a solvent approximates a linear function of the amount of solute added, and in both cells and serum nearly all the variable solute is protein. We may, therefore, with approximate accuracy, write

$$(25) \quad \frac{V_c}{V_b} = G_b \{ (\text{H}_2\text{O})_c + m (\text{Hb}) \}$$

$$(26) \quad \frac{V_s}{V_b} = G_b \{ (\text{H}_2\text{O})_s + n (\text{P})_s \}$$

where  $G_b$  is the specific gravity of the blood, with water at the same temperature as unity;  $m$  and  $n$  represent the volumes occupied in solution by a unit of cell and serum protein, respectively. When Hb and  $P_s$  are expressed in gram units,  $m$  and  $n$  both have values somewhat less than 1, since a gram of protein occupies somewhat less than 1 cc. volume. For horse blood we have found  $m = 0.90$  and  $n = 0.85$  when Hb and  $P_s$  are expressed as grams of protein. When Hb is expressed in millimols of  $\text{O}_2$  capacity,  $m = 0.90 \times 0.0167 = 0.015$ .

The introduction of the factor  $G_b$  into Equations 25 and 26 is in accordance with the following considerations. If we assume that

the blood proteins have the same coefficient of expansion as water, which assumption for the slight temperature effects involved can produce no significant error, then the volumes of cells and serum, respectively, per *kilo* of blood will be represented by the quantities  $\frac{(H_2O)_c + m(Hb)}{D_{H_2O}}$  and  $\frac{(H_2O)_s + n(P)_s}{D_{H_2O}}$ , respectively, ( $D_{H_2O}$  = density of water at the temperature of measurement, that of water at  $4^\circ$  being unity). In order to change the volumes per kilo of blood to volumes per liter, we multiply the former by  $D_b$ , the density of blood, with water at  $4^\circ$  as unity. But  $D_b = G_b \times D_{H_2O}$ .

Multiplying therefore  $\frac{(H_2O)_c + m(Hb)}{D_{H_2O}}$  and  $\frac{(H_2O)_s + n(P)_s}{D_{H_2O}}$  by  $G_b D_{H_2O}$  we obtain the volume values indicated in Equations 25 and 26.

Introducing the numerical values for  $m$  and  $n$  in Equations 25 and 26 we obtain

$$(27) \quad \frac{V_c}{V_b} = G_b \left\{ (H_2O)_c + 0.015 (Hb) \right\}$$

(Hb) being expressed in millimols of oxygen capacity per kilo of blood, and

$$(28) \quad \frac{V_s}{V_b} = G_b \left\{ (H_2O)_s + 0.85 (P)_s \right\}$$

(P)<sub>s</sub> being expressed as grams of serum protein per gram of blood (not, for this equation, as grams per kilo of blood).

The value of  $G_b$  and  $(H_2O)_b$ , constant for a given blood, may be estimated for normal horse blood as

$$(29) \quad G_b = 1.027 + 0.0037 (Hb)$$

and

$$(30) \quad (H_2O)_b = 0.914 - 0.015 (Hb)$$

The numerical constants in Equations 29 and 30 are obtained as described in connection with Equations 16 and 17, the first constant in each equation representing the  $G_b$  or  $(H_2O)_b$  value for normal serum, the second constant representing the change per unit increase in (Hb).

The agreement of the  $(H_2O)_s$  and  $(H_2O)_c$  values calculated at varying pH by Equations 23 and 24 with our observed values is

indicated by the tables in the experimental part of this paper, and is summarized in Fig. 13.

Warburg (10) has estimated the changes in cell volume with varying pH by measuring the oxygen capacity of the cells. The number of his determinations is sufficiently large to permit the plotting of an average curve by means of which the errors, that appear inherent in any method thus far used in estimating the small percentage changes in cell volume involved, are to a considerable extent neutralized. Warburg expresses his results in volume of cells at varying pH, compared with the volume at pH.

TABLE II.  
*Calculated Effect of pH Change on Water Distribution Compared with Effect Observed by Warburg.*

Blood constants estimated from hemoglobin content.

$$\begin{array}{ll} (\text{Hb}) = 11.3 \text{ observed.} & (\text{B})_s = 54.2 \text{ from Equation 16.} \\ (\text{P})_s = 0.0279 \text{ from Equation 17.} & G_b = 1.069 " " 29. \\ (\text{B})_c = 67.8 " " 15. & (\text{H}_2\text{O})_b = 0.745 " " 30. \end{array}$$

$\text{pH}_s$	$-\log r$ (from Fig. 1).	$\text{pH}_c$	$(\text{BP})_s$	$(\text{BP})_c$	$\frac{V_c}{V_b}$	Volume of cells in per cent of their volume at pH 6.8.	
						Calculated by Equation 27.	Observed by Warburg.
6.8	0.04	6.76	4.2	6.5	0.640	100.0	100.0
7.0	0.07	6.93	4.6	13.4	0.631	98.6	97.4
7.2	0.10	7.10	5.0	20.4	0.621	97.0	95.2
7.4	0.14	7.26	5.3	26.9	0.611	95.5	93.7
7.6	0.18	7.42	5.7	33.4	0.602	94.0	92.2
7.8	0.22	7.58	6.1	40.0	0.591	92.3	90.5

6.5. In Table II we have calculated for a blood, of the average hemoglobin content ( $(\text{Hb}) = 11.3$  millimolar) of the bloods used by Warburg, the change in cell volume as estimated by Equation 27. We have used as the unit of comparison the volume at  $\text{pH}_s$  6.8 instead of  $\text{pH}_s$  6.5, for the reason that both our experimental data and Warburg's are less complete and appear less certain below pH 6.8 than above it.

The changes observed by Warburg agree with those calculated by Equation 27 within the limit of experimental error, as do the changes observed by us, except in one experiment (No. 2). War-

burg's observed changes tend to exceed the calculated, while those determined in our experiments tend to fall short where they deviate from the calculated. The available data appear to agree with the predicted values as closely as the limitations of present accuracy in water determinations justify expecting.

In Fig. 2 the relative cell volume changes resulting from pH variations in oxygenated blood, as calculated by Equation 27,

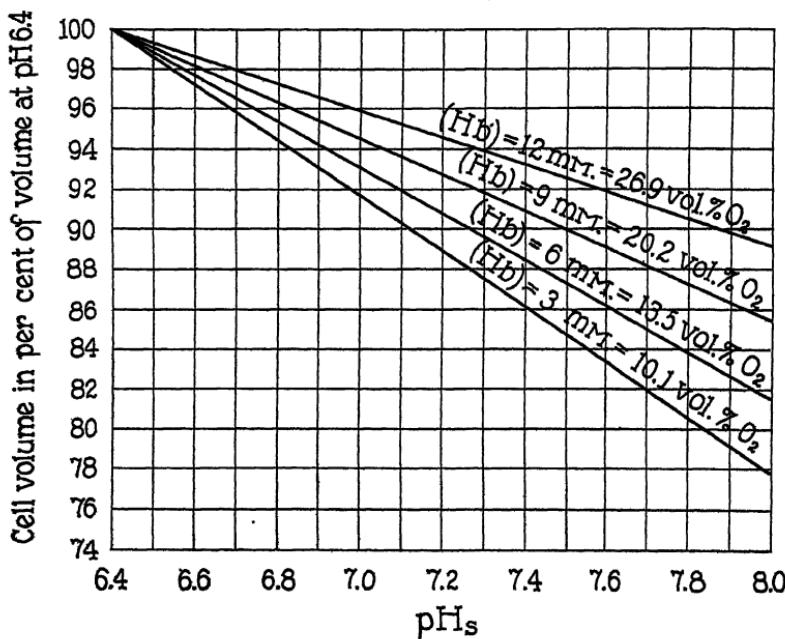


FIG. 2. Cell volumes calculated by Equations 24 and 27 for blood of average serum and cell composition observed in our experiments.

are shown for bloods of varying hemoglobin content. The percentage cell volume change caused by a given pH shift is greatest when the ratio cells: serum is least (hemoglobin lowest), because the concentration or dilution of serum which results from the water exchange, and tends to diminish the latter, is least when the relative amount of cells is smallest.

*Illustration of the Effect of CO<sub>2</sub> Tension Changes on the Electrolyte and Water Distribution of Oxygenated Blood.*

To illustrate the processes involved we may simplify conditions by ignoring minor factors; *viz.*, the slight amounts of diffusible anions other than Cl' and HCO<sub>3</sub>', the osmotic and base-binding powers of the serum proteins, and the osmotic effect of the hemoglobin. We shall assume the cells to contain only base, hemoglobin, Cl, and HCO<sub>3</sub>, and the serum to be a simple solution of

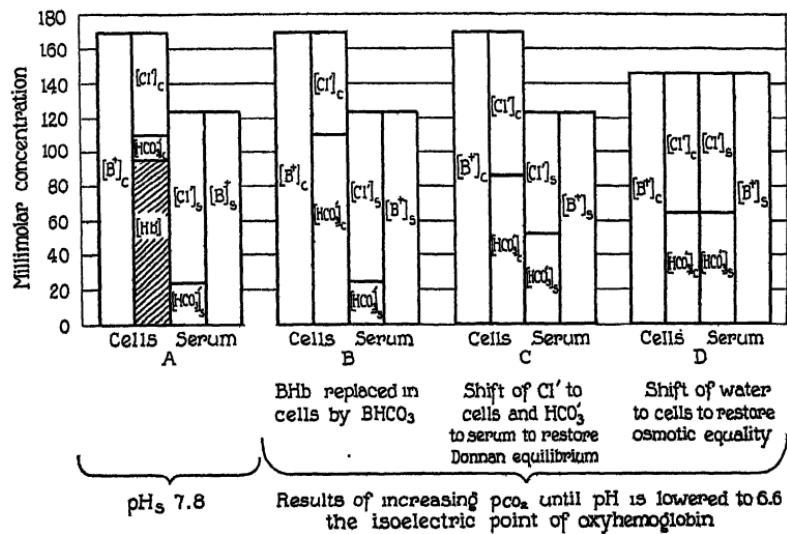


FIG. 3.

bicarbonate and chloride. Equation 10 under these conditions becomes simplified to

$$r = \frac{[H^+]_s}{[H^+]_c} = \frac{[Cl']_c}{[Cl']_s} = \frac{[HCO_3']_c}{[HCO_3']_s} = 1 - \frac{[BHb]_c}{2([BCl]_s + [BHCO_3]_s)}$$

We shall assume, first that the CO<sub>2</sub> tension is so low that pH<sub>c</sub> = 7.8, then that it is raised so that pH falls to 6.6. According to Van Slyke, Hastings, Heidelberger, and Neill (1), the alkali bound by oxyhemoglobin is indicated by the equation [BHb] = 2.65 [Hb] (pH = 6.6). Assuming [Hb]<sub>s</sub> = 30 mm., we therefore calculate at pH<sub>c</sub> = 7.8 that [BHb] = 95, and at pH<sub>c</sub> = 6.6 that [BHb] = 0.

In Fig. 3 we have indicated the concentrations of the positively and negatively charged ions in the cells and serum by the areas assigned to each ( $[Hb']$  is indicated in terms of alkali equivalents bound). The concentrations of the osmotically active ions are indicated by clear areas, while that of the (relatively) osmotically inactive  $[Hb']$  is indicated by a shaded area. For simplicity it is assumed that the ionization of each electrolyte is complete. It is also assumed that at the beginning (Fig. 3 A) the water content of the blood is half in the cells, half in the serum.

The amounts of hemoglobin, base, chloride, and bicarbonate indicated are about those found in normal horse blood, except that the difference between  $[B]_c$  and  $[B]_s$  in Fig. 3 A is somewhat exaggerated as a result of ignoring the base bound by the serum proteins and the osmotic effect of the hemoglobin.

The conditions indicated in the four diagrams of Fig. 3 are the following:

A. The conditions represented conform to the three basic laws: (1) in both cells and serum the positive and negative ions balance; (2) the ratios  $\frac{[Cl']_c}{[Cl']_s}$  and  $\frac{[HCO_3']_c}{[HCO_3']_s}$  are equal, and conform to the simplified form of Equation 10 given above; and (3) the osmolar concentrations obtained by adding  $[B^+] + [Cl'] + [HCO_3']$  are equal in serum and cells respectively.

B. Increase of  $CO_2$  tension has lowered the  $pH_c$  to 6.6, the isoelectric point of oxyhemoglobin. The result is that all the base formerly bound by hemoglobin as  $BHb$  has shifted to  $BHCO_3$ ,  $HCO_3'$  replacing  $Hb'$ . In Fig. 3 B, however, only the first of the three laws is conformed with. Positive and negative charges balance, but the greatly increased concentration of  $HCO_3'$  in the cells obviously makes  $\frac{[HCO_3]_c}{[HCO_3]_s} > \frac{[Cl]_c}{[Cl]_s}$ , contrary to Donnan's law. The  $HCO_3'$  increase in the cells also causes the osmolar concentration there to exceed that in the serum. The system is not in equilibrium.

C. To restore electrolyte distribution to conformity with Donnan's law,  $Cl'$  has migrated from serum to cells, and  $HCO_3'$  in the reverse direction until again  $\frac{[HCO_3]_c}{[HCO_3]_s} = \frac{[Cl]_c}{[Cl]_s}$ .

D. To restore also osmotic equilibrium, water has migrated from serum to cells until the osmolar concentrations in both are equal. Impermeability of the cell membranes to cations prevents diffusion of  $\text{BCl}$  and  $\text{BHCO}_3$  from cells to serum to assist in the restoration of osmolar equality. It must all be accomplished by water transfer. The system is now in equilibrium again.

The processes represented here, for the sake of analysis, as though occurring in successive steps must in reality occur simultaneously.

The somewhat more complex changes actually occurring in blood, where the alkali-binding power of the serum proteins and the osmotic pressure of the hemoglobin enter as appreciable,

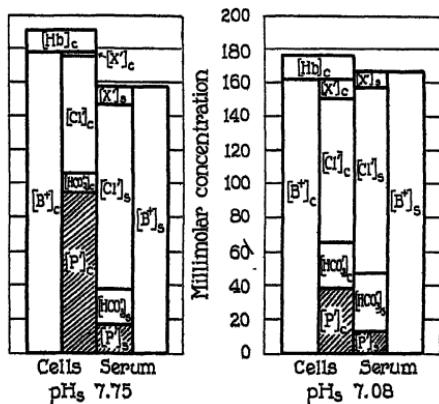


FIG. 4. Relationships observed in Experiment 3.

though minor factors, are indicated by Fig. 4, which represents data obtained in Experiment 3 on defibrinated blood.  $\text{X}'$  is used to indicate the undetermined anions.

#### *Calculation of the Electrolyte and Water Changes in Blood During the Respiratory Cycle.*

In Table IIIa we have calculated the changes that, according to our data, may be expected to accompany the  $\text{CO}_2$  and  $\text{O}_2$  changes of ordinary respiration. For a blood of  $(\text{Hb}) = 9.0 \text{ mm. per kg.}$  the other blood constants  $(\text{B})_a$ ,  $(\text{B})_s$ ,  $(\text{P})_s$ ,  $G_b$ , and  $(\text{H}_2\text{O})_b$  are calculated from Equations 15, 16, 17, 29, and 30. The  $(\text{Cl})_b$  content is taken arbitrarily at 76, and the  $(\text{BHCO}_3)_b$  in arterial blood

TABLE III. a.  
*Estimation of the Changes Occurring in the Normal Respiratory Cycle.*

	Arterial.		Venous.		Changes.	
	Serum.	Cells.	Serum.	Cells.	Whole blood.	Whole blood.
(Hb)	mm. per kg. blood.....	0.0	9.0	9.0	9.0	0.0
(P) <sup>a</sup>	gm. " "	37.0	0.0	37.0	0.0	0.0
(B)	m.-Eq. " "	73.0	54.0	127.0	54.0	127.0
(H <sub>2</sub> O)	kg. " "	0.464	0.316	0.780	0.460	0.780
(Cl)	m.-Eq. " "	51.2	24.8	76.0	50.0	76.0
(BP)	" "	6.62	22.01	28.63	6.54	19.87
(BHCO <sub>3</sub> )	" "	12.79	6.21	19.00	13.96	7.26
(H <sub>2</sub> CO <sub>3</sub> )	" "	0.626	0.426	1.052	0.733	0.510
Total CO <sub>2</sub>	" "	13.42	6.63	20.05	14.69	7.77
Combined O <sub>2</sub> mm.	" "	0.0	8.8	8.8	0.0	6.0
Dissolved O <sub>2</sub>	" "	0.0	0.14	0.14	0.03	0.03
Total O <sub>2</sub>	" "	0.0	8.94	8.94	6.03	6.03
pco <sub>2</sub>	mm. Hg " "	41.6	41.6	49.2	49.2	49.2
pO <sub>2</sub>	" "	100.0	100.0	38.0	38.0	38.0
pH.....		7.43	7.283	7.40	7.272	7.272
Volume l. per l. blood.....		0.525	0.475	1.000	0.521	0.479
r ratio.....						
(r) " .....				0.713		0.746
				0.485		0.519
Respiratory quotient $\frac{\Delta \text{CO}_2}{\Delta \text{O}_2}$ .....						
						0.83

TABLE III b.  
*Changes from Arterial to Venous Blood.*

	Total CO <sub>2</sub> .	Cell volume.	Serum Cl.	Proportion of total CO <sub>2</sub> increase in venous cells.	Proportion of total CO <sub>2</sub> increase in venous serum.
	mM. per kg. blood	per cent of blood vol.	mM. per l. serum	per cent	per cent
Calculated from data of Table III a on horse blood.....	+2.4	+0.4	-1.2	49.3	50.7
Observed by Dusy and Beckmann (20) in dog blood.....	+1.9	+0.35	-1.38	52.4	47.6

is taken at 19.0. The combined oxygen ( $O_2$ ) is assumed to be 8.8 mm. in arterial blood, and 6.0 in venous blood, and  $pH_s$  is assumed to be 7.43 in arterial blood, and 7.40 in venous blood. From these  $pH_s$  values the  $r$  and  $pH_c$  values are estimated from the curves of Fig. 1.

The  $(BP)_c$  and  $(BP)_s$  values are calculated from Equations 57 and 54 respectively (see experimental part of the paper). The increment in  $(BHCO_3)$  when the arterial blood becomes venous is estimated equal to the decrease in  $(BP)_c + (BP)_s$ , the increase in bicarbonate being due to alkali taken from the other buffers to combine with  $H_2CO_3$ . (The diffusible buffers other than carbonates are neglected, as their effect is relatively slight.)

The distribution of water between serum and cells is calculated from Equations 23 and 24.

The distribution of Cl and  $HCO_3$  is calculated as follows:

Letting A represent either Cl or  $HCO_3$ , we have from Equation 9,  $\frac{[A]_c}{[A]_s} = r$ . But  $[A]_c = \frac{(A)_c}{(H_2O)_c}$  and  $[A]_s = \frac{(A)_s}{(H_2O)_s}$ . Hence  $\frac{(A)_c}{(A)_s} = r \frac{(H_2O)_c}{(H_2O)_s}$ . We represent the factor  $r \frac{(H_2O)_c}{(H_2O)_s}$  by  $(r)$ . Then,  $(A)_c = (r)(A)_s, \frac{(A)_c}{(A)_c + (A)_s} = \frac{(r)}{1 + (r)}$ , and  $\frac{(A)_s}{(A)_c + (A)_s} = \frac{1}{1 + (r)}$ . From the values of  $(r)$ , and of  $(A)_c + (A)_s$ , which is the total  $(Cl)_s$  or  $(HCO_3)_s$ , we accordingly calculate  $(A)_c$  and  $(A)_s$ .

Using the  $pK'$  value 6.12 (see "Calculations" in experimental part), we calculate the  $(H_2CO_3)_s$  and  $(H_2CO_3)_c$  from the corresponding  $(BHCO_3)$  and  $pH$  values by a rearrangement of Hasselbach's (21) equation as

$$\log (H_2CO_3) = pK' - pH + \log (BHCO_3)$$

The  $CO_2$  tension is calculated as

$$p_{CO_2} = \frac{(H_2CO_3)}{0.0324 \times (H_2O)}$$

since  $[H_2CO_3] = \frac{(H_2CO_3)}{(H_2O)} = 0.0324 p_{CO_2}$  (see "Calculations" in experimental part).

The oxygen tensions have been approximated from Barcroft's curves on human blood (37). Their degree of accuracy when applied

to horse blood data is uncertain, but we have used them to indicate at least the magnitude of the changes.

In short the serum pH and the oxygen contents of the venous and arterial bloods have been arbitrarily chosen at about the observed normal values, and the other data have been calculated from them.

It will be noted that in Table IIIa the figures are given in terms of millimols or milli-equivalents of serum or cell constituents per kg. of whole blood, not per kg. or per liter of the serum or cells. The concentration values in the serum and cells in terms of solute: water may be obtained from the figures per kilo of blood by dividing the latter by the water content; e.g.,  $\frac{\text{serum Cl}}{\text{serum H}_2\text{O}} = \frac{\text{serum Cl}}{\text{kg. blood}} \div \frac{\text{serum H}_2\text{O}}{\text{kg. blood}}$ . Similarly  $\frac{\text{serum Cl}}{\text{liter serum}} = \frac{\text{serum Cl}}{\text{liter serum}} \div \frac{1.05 \text{ kg. blood}}{\text{liter blood}}$ .

In Table IIIb we have compared the average arterial-venous differences observed in dog blood by Doisy and Beckmann (20) with the changes calculated from Table IIIa. The observed figures are of the same order of magnitude as those calculated.

In Fig. 5 we have indicated the relationships on a D'Ocagne nomogram, of a type that was devised by L. J. Henderson (private communication about 2 years ago). A straight line, drawn across the chart, and cutting the lines representing oxygen and CO<sub>2</sub> tensions at any given points, cuts the lines representing (BHCO<sub>3</sub>)<sub>b</sub>, (HbO<sub>2</sub>), pH<sub>s</sub>, pH<sub>c</sub>, etc., at points indicating the values these respective quantities have under the given PCO<sub>2</sub> and PO<sub>2</sub>. Such a line can be drawn because all the other variables in a given blood are dependent on these two. Over the range used, the chart is quite exact. The construction of such blood charts will be discussed in a later paper by Henderson.

#### *Electrolyte Distribution between Blood Serum and Transudates as a Function of the Alkali Bound by the Proteins.*

Loeb, Atchley, and Palmer (22) have performed experiments indicating that the membranes separating the blood serum from the fluids in the body cavities and intercellular spaces have the same permeabilities as collodion for the substances present.

Under these conditions the Donnan distribution would require expression by an equation including Na and K among the diffusible ions, instead of excluding them, as does Equation 3. Expressing the distribution ratio of monovalent ions between serum and fluid as  $r_{sf}$ , the relationship would theoretically be

$$(31) \quad r_{sf} = \frac{[A']_s}{[A']_f} = \frac{[B^+]_f}{[B^+]_s} = \frac{[H^+]_f}{[H^+]_s}$$

when  $\frac{[A']_s}{[A']_f}$  indicates the ratio of the osmotic activity of any monovalent anion, or sum of anions, in the serum to the osmotic activity of the same ion or ions in the fluid, while  $\frac{[B^+]_f}{[B^+]_s}$  has a similar significance for the cations. If in place of  $[B^+]_s$  and  $[B^+]_f$  we substitute their values from Equations 1 and 2 we obtain

$$(32) \quad r_{sf} = \frac{[A']_f + [BP]_f}{[A']_s + [BP]_s}$$

If we substitute  $\frac{A_s}{(r_{sf})}$  for  $A_f$ , and solve for  $r_{sf}$  we obtain

$$(33) \quad r_{sf} = \frac{[BP]_f + \sqrt{[BP]_f^2 + 4 [A]_s ([A]_s + [BP]_s)}}{2 ([A]_s + [BP]_s)}$$

We have recalculated in Table IV Loeb, Atchley, and Palmer's data, transposing the concentrations from  $\frac{\text{solute}}{\text{volume}}$  to  $\frac{\text{solute}}{\text{water}}$  ratios by estimating the grams of water per liter to be 990 - 0.8 P, where P represents grams of protein per liter. (This may be taken as a fairly close approximation, unless abnormal amounts of fat or other solids are present.) The  $[BP]_s$  and  $[BP]_f$  values are calculated on the assumption that the proteins of human serum at pH 7.4 bind the same amount of alkali per gram as the proteins of horse serum (the slight difference in pH between plasma and fluid may be neglected). At this pH the formula (Equation 54)  $[BP] = 0.068 [P]$  ( $\text{pH} - 4.80$ ) becomes  $[BP] = 0.177 [P]$ . The arterial  $\text{HCO}_3^-$  values are estimated by subtracting 2 mm. per liter from the values found in the venous serum.

The estimated  $[\text{Cl}]_s : [\text{Cl}]_f$  ratios found coincide with the calculated  $r_{sf}$  values nearly within the limit of experimental error.

The  $[HCO_3]_s : [HCO_3]_v$  ratios are all higher than the calculated  $r_{sf}$  when the venous values for  $[HCO_3]_s$  are used; but the estimated arterial values for  $[HCO_3]_s$  yield  $[HCO_3]_s : [HCO_3]_v$  ratios which agree with the calculated  $r_{sf}$  as closely as could be expected, when the possible magnitude of the error involved in assuming a constant difference between arterial and venous  $CO_2$ , is considered.

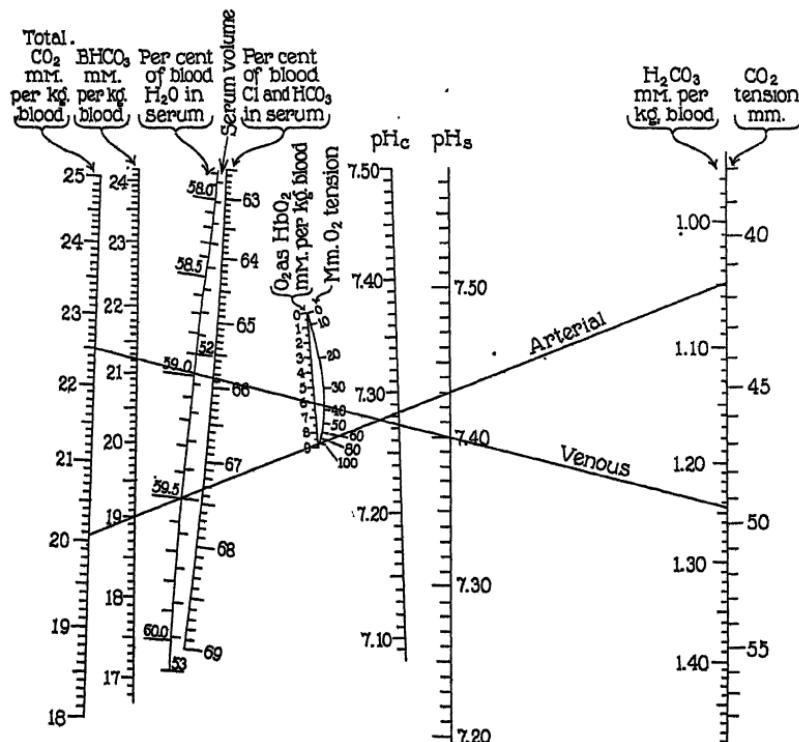


FIG. 5. D'Ocagne-Henderson nomogram showing calculated relationships for arterial and venous blood of average serum and cell composition observed in our experiments.

The  $[Na]_f : [Na]_s$  ratios agree, in six out of seven cases, with the calculated  $r_{sf}$  values within the rather wide limit of error assigned by the authors to the Na determination. The  $[K]_f : [K]_s$  ratios are altogether lower than the calculated  $r_{sf}$ , and are very irregular. The source of the deviation and irregularity of the K ratios is at

TABLE IV.  
Observed Electrolyte Distribution between Blood Serum and Serum Cavity Fluids Compared with Distribution Ratio Calculated from Base Bound by Proteins. Calculated from the Data of Loeb, Attley, and Palmer.

Subject.	Cl	K	Co	D	P	McA	H
Extravascular fluid.....	Ascitic.	Ascitic.	Ascitic.	Chest.	Ascitic.	Ascitic.	Chest.
Serum protein, gm./l.....	68	52	70	50	60	73	71
Fluid " "	9	8	9	6	33	45	56
Estimated serum H <sub>2</sub> O, kg./l.....	0.986	0.948	0.934	0.950	0.942	0.932	0.933
" fluid " "	0.982	0.984	0.983	0.985	0.984	0.954	0.945
[P], gm./kg. H <sub>2</sub> O.....	73	55	75	53	64	78	76
[P] <sub>J</sub> " "	9	8	9	6	24	47	59
[BP] <sub>s</sub>	12.9	9.7	13.3	9.4	11.3	13.9	13.5
[BP] <sub>J</sub>	"	1.5	1.4	1.5	1.0	6.0	9.3
[Cl] <sub>s</sub>	"	"	"	"	"	122.5	113.4
[Cl] <sub>J</sub>	"	"	"	"	"	121.5	118.0
Venous [HCO <sub>3</sub> ] <sub>s</sub>	"	29.4	25.1	28.3	28.4	17.9	30.5
Estimated arterial [HCO <sub>3</sub> ] <sub>s</sub>	"	27.4	23.1	26.3	26.4	15.9	28.5
[HCO <sub>3</sub> ] <sub>J</sub>	"	"	"	"	"	27.0	16.8
[Na] <sub>s</sub>	"	"	133.2	141.1	120.0	157.1	148.5
[Na] <sub>J</sub>	"	"	140.8	128.8	141.0	149.8	146.0
[K] <sub>s</sub>	"	"	4.5	5.0	2.9	3.7	5.0
[K] <sub>J</sub>	"	"	2.4	2.0	2.4	1.7	3.2
[Cl] : [Cl] <sub>J</sub> ratio.....		0.97	0.99	0.98	0.96	0.98	0.96
Venous [HCO <sub>3</sub> ] <sub>s</sub> : [HCO <sub>3</sub> ] <sub>J</sub>	"	1.10	1.04	1.08	1.05	1.07	1.02
Estimated arterial " "	"	1.02	0.95	1.01	0.98	0.95	0.96
[Na] <sub>J</sub> : [Na] <sub>s</sub>	"	1.06	0.91	1.17	0.95	0.98	0.99
[K] <sub>J</sub> : [K] <sub>s</sub>	"	0.53	0.40	0.83	0.46	0.64	0.66
Calculated r <sub>st</sub> .....		0.95	0.97	0.96	0.96	0.98	0.99

present uncertain. Considering the minute amounts of K present, it appears possible that the irregularities may lie in the micro method used for the determination.

The irregularity of the potassium ratios, and the necessity for using estimated water and arterial  $\text{HCO}_3$  values, make it impossible to consider the presence of a Donnan equilibrium between blood serum and edema fluid as quantitatively demonstrated with satisfactory accuracy. It appears probable nevertheless that the degree of agreement found between the calculated  $r_{sf}$  values and the ratios for Cl,  $\text{HCO}_3$  (arterial), and Na is more than fortuitous; that it affords support for Loeb, Atchley, and Palmer's conclusion that "the relationships between serum and edema fluid result from a simple membrane equilibrium, influenced in part by the proteins present."

If the membranes separating blood serum from other extracellular fluids are permeable to all electrolytes present in amounts of quantitative importance except protein, it follows from the Donnan theory that the serum, containing more protein than the other fluids, must when at equilibrium with them show a positive osmotic pressure. While the basic equations of the form of Equations 1 and 2, and of Equation 3 modified to include Na, hold for such a system, Equation 4 and its derivatives expressing osmolar equality do not, so long as the serum volume is limited. The preponderance of the osmolar concentration even of the diffusible ions, on the side containing non-diffusing ions, when the latter are entirely on one side of the membrane and infinite volume change is excluded, has been theoretically shown by Procter and Wilson (15).

If the non-diffusible electrolyte (protein) also has a measurable attraction for water, the osmotic preponderance on its side of the membrane is still further increased. If serum and a transudate relatively poor in protein are separated by membranes permeable to all the non-protein ions present in quantitatively important concentrations, *viz.*  $\text{Na}^+$ ,  $\text{Cl}'$ , and  $\text{HCO}_3'$ , but impermeable to the protein, we may therefore expect the serum to exhibit a higher osmotic pressure than the edema fluid. With the osmotic pressure tending to draw water into the serum, it appears that forces other than that of osmotic pressure are involved in the passage of fluid in the direction from the blood to the serous cavities and intercellular spaces.

*Effect of the Membrane on the Determination of the Blood pH by the Dialysis Method.*

Levy, Rowntree, and Marriott (23) have devised a method for blood pH determination in which the blood is dialyzed against 0.8 per cent NaCl solution, and the pH of the dialysate is determined colorimetrically. This method has later been revised by Dale and Evans (24), who added precautions to prevent loss of CO<sub>2</sub>. It is evident that, because protein is present only on one side of the membrane, the Donnan effect must introduce some error into such a method. On the basis of our data defining the amount of base bound by the serum proteins at physiological pH ranges, it appears possible to estimate the approximate magnitude of this error.

In the short time in which measurable change of pH in the dialysis fluid ceases (5 minutes) the volume change may be neglected, and we may consider the system the same as that discussed in the preceding section, *viz.* one in which the volumes are fixed, the osmotic pressures are not necessarily equal, and the solutes, except protein, are diffusible through the membrane. Equation 33 then applies, but is simplified because [BP]<sub>f</sub> is zero, since there is no protein in the fluid outside the membrane. In place of Equation 33 we therefore have, when equilibrium among the electrolytes is reached:

$$(34) \quad r_{sf} = \frac{[\text{H}^+]_f}{[\text{H}^+]_s} = \sqrt{\frac{[\text{A}]_s}{[\text{A}]_s + [\text{BP}]_s}}$$

whence,

$$(35) \quad \text{pH}_f - \text{pH}_s = \frac{1}{2} \log \frac{[\text{A}]_s + [\text{BP}]_s}{[\text{A}]_s}$$

If we take A<sub>s</sub> = 150 mm., [BP]<sub>s</sub> = 14, which are fairly close to the usual values, we obtain

$$\text{pH}_f - \text{pH}_s = \frac{1}{2} \log \frac{164}{150} = 0.02$$

It appears, therefore, that when serum (or blood) is dialyzed against a solution of approximately the same salt concentration, we may expect a pH in the solution about 0.02 greater than in the serum.

*Relationship between Hasselbalch's Constant for Whole Blood and the K' s Value for Serum.*

As Warburg has pointed out, since blood is a heterogeneous system, and cell and serum pH and  $\text{BHCO}_3$  values differ, the constant  $K'_B$  of the equation

$$(36) \quad K'_B = [H^+]_s \frac{[BHCO_3]_b}{[H_2CO_3]_b}$$

used by many authors since Hasselbalch (20) in applying Henderson's equation to whole blood, is really an approximate constant, which contains within itself corrections, hitherto empirical, for the differences that exist between cells and serum. (Hasselbalch used the symbol  $K_1$ . We have used  $K'_B$  in order to distinguish it from  $K'_s$ .) The relationship of  $K'_B$  to the constant  $K'_s$  is shown as follows:

$$(37) \quad K'_s = [H^+]_s \frac{[BHCO_3]_s}{[H_2CO_3]_s}$$

Dividing Equation 36 by Equation 37 we obtain

$$(38) \quad \frac{K'_B}{K'_s} = \frac{[BHCO_3]_b}{[BHCO_3]_s} \times \frac{[H_2CO_3]_s}{[H_2CO_3]_b}$$

If concentrations are expressed in the terms of solute: water ratios used in this paper  $[H_2CO_3]_s$  and  $[H_2CO_3]_b$  are equal (see discussion of  $[H_2CO_3]$  calculations on pages 824 and 825). Under these conditions  $\frac{[H_2CO_3]_s}{[H_2CO_3]_b} = 1$ , and Equation 38 simplifies to

$$(39) \quad \frac{K'_B}{K'_s} = \frac{[BHCO_3]_b}{[BHCO_3]_s}$$

Equation 38 may be used for calculating  $\frac{K'_B}{K'_s}$  from the  $\text{CO}_2$  contents of whole blood and true serum, whatever unit of concentration is employed, and Equation 39 may be used with the solute: water ratio adopted as the concentration unit in this paper.

The relationship of the  $\frac{K'_B}{K'_s}$  ratio to the electrolyte and water distribution in the blood is shown as follows:

Since the total bicarbonate in 1,000 gm. of blood is equal to the sum of that in the serum plus that in the cells, we have

$$(40) \quad [\text{BHCO}_3]_b \times (\text{H}_2\text{O})_b = [\text{BHCO}_3]_s \times (\text{H}_2\text{O})_s + [\text{BHCO}_3]_c \times (\text{H}_2\text{O})_c$$

whence

$$(41) \quad [\text{BHCO}_3]_b = [\text{BHCO}_3]_s \frac{(\text{H}_2\text{O})_s}{(\text{H}_2\text{O})_b} + [\text{BHCO}_3]_c \frac{(\text{H}_2\text{O})_c}{(\text{H}_2\text{O})_b}$$

but  $[\text{BHCO}_3]_c = r [\text{BHCO}_3]_s$  (see Equation 3).

Substituting in Equation 41 the above value for  $[\text{BHCO}_3]_c$ , we obtain

$$(42) \quad [\text{BHCO}_3]_b = [\text{BHCO}_3]_s \frac{(\text{H}_2\text{O})_s + r (\text{H}_2\text{O})_c}{(\text{H}_2\text{O})_b}$$

Substituting this value for  $[\text{BHCO}_3]_b$  in Equation 39, we obtain

$$(43) \quad \frac{K'_B}{K'_s} = \frac{(\text{H}_2\text{O})_s + r (\text{H}_2\text{O})_c}{(\text{H}_2\text{O})_b}$$

In logarithmic terms, using  $\text{pK}'_B = -\log K'_B$ ,  $\text{pK}'_s = -\log K'_s$ , Equation 43 becomes

$$(44) \quad \text{pK}'_B - \text{pK}'_s = \log \frac{(\text{H}_2\text{O})_b}{(\text{H}_2\text{O})_s + r (\text{H}_2\text{O})_c}$$

The values for  $(\text{H}_2\text{O})_s$  and  $(\text{H}_2\text{O})_c$  are obtainable from Equations 23 and 24, and the values of  $r$  from Equations 10 and 12.

The values of  $\text{pK}'_B - \text{pK}'_s$  calculated by Equation 44 at varying  $\text{pH}_s$  and for bloods of varying hemoglobin content are given in Fig. 6a.

In Fig. 6b Dr. Hastings has prepared a D'Ocagne line chart which facilitates interpolations of the  $\text{Hb}$ ,  $\text{pH}_s$ , and  $\text{pK}'_B - \text{pK}'_s$ , relationships. The chart was constructed by laying off the  $(\text{Hb})$  and  $\text{pK}'_B - \text{pK}'_s$  values logarithmically on their respective lines, and locating each  $\text{pH}_s$  point at the intersection of the lines, connecting the 3, 6, 9, and 12 ( $\text{Hb}$ ) points with the  $\text{pK}'_B - \text{pK}'_s$  points calculated from the given  $\text{pH}_s$  and the respective  $(\text{Hb})$  values. Over the  $\text{Hb}$  range 3 to 9 mm., the chart indicates

values for  $pK'_B - pK'_s$  differing from those calculated from Equation 44 by only 0.001. A straight line drawn across the chart indicates the  $pK'_B - pK'_s$  value corresponding to the Hb and pH<sub>s</sub> points intersected.

The curves are of the same form as those obtained in a different manner by Warburg.<sup>6</sup> The values for  $pK'_B - pK'_s$ , indicated by our curves are 0.01 to 0.02 higher than Warburg's at ordinary physiological pH and (Hb) ranges.

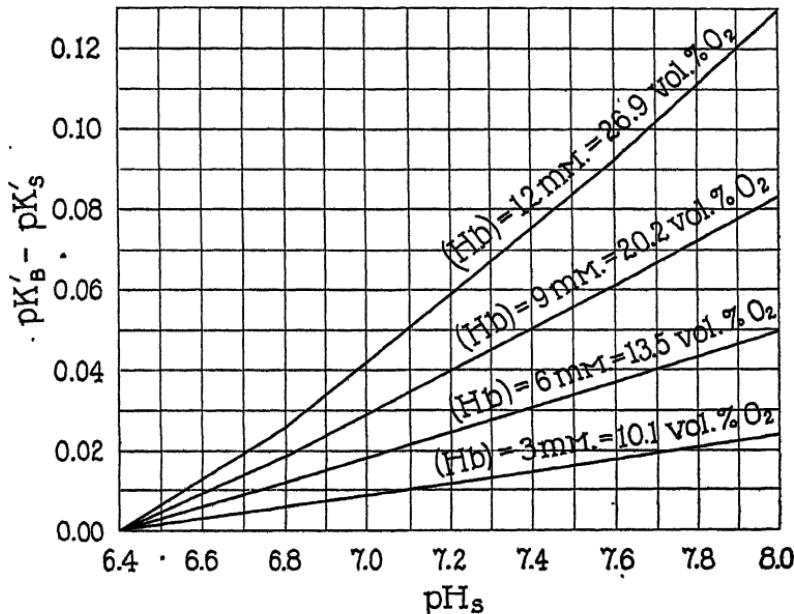


FIG. 6a.

Peters, Bulger, and Eisenman (25) in a paper appearing since our work was completed, have determined the value of  $pK'_B - pK'_s$  (for which they use the symbol  $\Delta pK_1$ ) in the most direct way from determinations of the CO<sub>2</sub> contents of whole blood and plasma in a large series of human bloods, saturated at known CO<sub>2</sub> tensions, their calculation being made practically as by our Equation 38. Their  $\Delta pK_1$  values, obtained within the

<sup>6</sup> See Warburg (10), p. 220.

range of their experiments (indicated by the solid lines of their Chart 4), when transposed from cell volume to (Hb) terms, deviate from our curves by 0.000 to 0.009. Considering the facts that their data represent the average from many human bloods, while ours are from the blood of one horse, and also that the difference between Bohr's solubility coefficients for  $\text{CO}_2$  used by

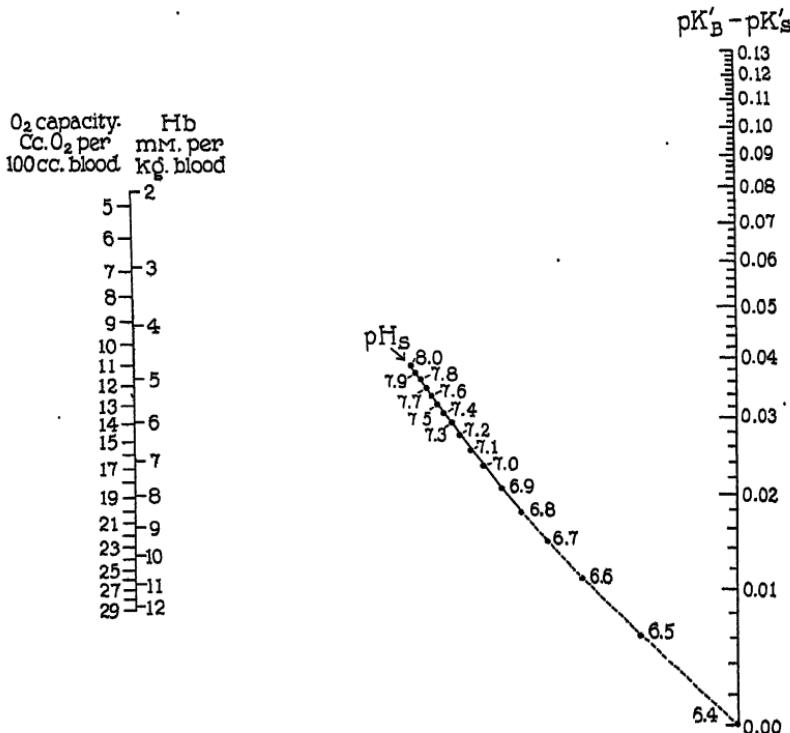


FIG. 6b.

them and the coefficients used by us appreciably affect the  $\Delta p\text{K}_1$  values, the agreement is close.

#### *Calculation of the $\text{CO}_2$ Absorption Curve and Buffer Value of Blood.*

Since, in a given blood submitted to varying  $\text{CO}_2$  tensions, the changes in bicarbonate are at the expense of alkali taken from or given to the other buffers (the proteins) we may represent the

total blood bicarbonate ( $\text{BHCO}_3$ )<sub>b</sub> as

$$(45) \quad (\text{BHCO}_3)_b = (\text{B})_{\text{P} + \text{HCO}_3} - (\text{BP})_b$$

where  $(\text{B})_{\text{P} + \text{HCO}_3}$  is a constant representing the base bound by proteins and bicarbonate together. (In normal horse blood, approximately  $(\text{B})_{\text{P} + \text{HCO}_3} = (\text{B})_b - 1.1 (\text{Cl})_b$ .) Since  $(\text{BP})_b = (\text{BP})_c + (\text{BP})_s$ , we may write Equation 45 as

$$(46) \quad (\text{BHCO}_3)_b = (\text{B})_{\text{P} + \text{HCO}_3} - \{(\text{BP})_c + (\text{BP})_s\}$$

By substituting from Equations 54 and 55 the approximate values for  $(\text{BP})_c$  and  $(\text{BP})_s$  in oxygenated blood, we obtain  $(\text{BHCO}_3)_b$  in terms of pH and protein content

$$(47) \quad (\text{BHCO}_3)_b = (\text{B})_{\text{P} + \text{HCO}_3} - \{3.6 (\text{Hb}) (\text{pH}_s - 6.6) + 0.068 (\text{P})_s (\text{pH}_s - 4.80)\}$$

The pH values may all be reduced to  $\text{pH}_s$  by substituting  $\text{pH}_s + \log r$  for  $\text{pH}_c$  (see Equation 18). We thus obtain

$$(48) \quad (\text{BHCO}_3)_b = (\text{B})_{\text{P} + \text{HCO}_3} - \{3.6 (\text{Hb}) (\text{pH}_s + \log r - 6.6) + 0.068 (\text{P})_s (\text{pH}_s - 4.80)\}$$

Between pH 7.0 and 7.8 we may approximate the value of  $\log r$  (see Fig. 1) with an error not greater than 0.01 by the linear formula,

$$(49) \quad -\log r = 0.21 \text{ pH}_s - 1.41$$

Substituting this value for  $\log r$  in Equation 48 we obtain

$$(50) \quad (\text{BHCO}_3)_b = (\text{B})_{\text{P} + \text{HCO}_3} - \{2.84 (\text{Hb}) (\text{pH}_s - 6.6) + 0.068 (\text{P})_s (\text{pH}_s - 4.80)\}$$

Equation 50 represents the  $\text{CO}_2$  absorption curve of oxygenated blood with  $(\text{BHCO}_3)_b$  and  $\text{pH}_s$  as ordinates.

(The same method of calculation may be used for reduced blood, 3.35 (Hb) being substituted for 3.6 (Hb), and  $-\log r = 0.18 \text{ pH}_s - 1.15$  for the above  $-\log r$  value. See Fig. 1 and Equation 55.)

By differentiating Equation 50 with respect to  $\text{pH}_s$  we obtain

the  $\frac{d(\text{BHCO}_3)_b}{dpH_s}$  value, which is the negative of the buffer values other than that of bicarbonate.

$$(51) \quad - \frac{d(\text{BHCO}_3)_b}{dpH_s} = 2.84 (\text{Hb}) + 0.068 (\text{P}_s)$$

That Equation 51 is approximately accurate for normal horse blood is indicated by comparison with the buffer values obtained on whole blood in the fourth paper (2) of this series and summarized in Table VII of that paper. Estimating the  $(\text{P}_s)$  values for the bloods from the hemoglobin contents by Equation 17 of the present paper, and calculating the buffer values by Equation 51, we obtain the results below.

Blood No. (Table VII Van Slyke, Hastings, and Neill, 2.)	$- \frac{d(\text{BHCO}_3)_b}{dpH_s}$	
	Observed.	Calculated by Equation 51.
1	23.72	23.9°
2	20.64	22.6
4	24.18	23.5
5	22.23	23.0
6	24.16	24.9

If the entire blood buffer system (as in laked blood) were at  $pH_s$ , we should have the  $(\text{BHCO}_3)_b$  represented by Equation 48 with  $\log r$  deleted from it, and differentiation would give

$$(52) \quad - \frac{d(\text{BHCO}_3)_b}{dpH_s} = 3.6 (\text{Hb}) + 0.068 (\text{P}_s)$$

The difference between Equations 51 and 52, *viz.* 0.76 (Hb), indicates the difference between the  $\frac{d(\text{BHCO}_3)_b}{dpH_s}$  value estimated on the assumption that all the blood buffers are at  $pH_s$ , and the value estimated on the assumption that the cell buffers are at  $pH_s = pH_s + \log r$ , or the approximate *increase in buffer value caused by laking blood*. (The value 0.76 (Hb) is only an approximation, because of the simplifying assumptions of linear functions that have been made above in deriving it.)

In the fourth paper of this series (2) the statement has been made that "The estimation from buffer constants obtained on hemoglobin solutions, of the buffer effect of hemoglobin in the blood involves the assumption that the confinement of hemoglobin within the red cells does not alter its buffer effect on the system. . . . The calculation of the relative part played by hemoglobin in the buffer effect of blood . . . is therefore presented with the reservation that slight corrections may be required because of the difference in pH between plasma and cell contents." It is obvious from the difference between Equations 51 and 52 above that confinement within the cells does alter the effect of the cell buffers on the system, and that the corrections anticipated as a possibility are of considerable significance. The point will be discussed in another paper with experimental data.

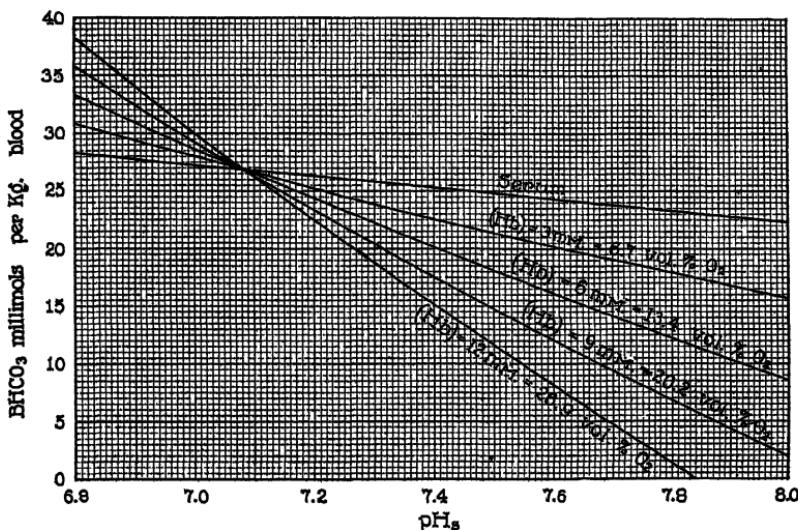


FIG. 7. Carbon dioxide absorption curves calculated by Equations 50 and 53 for oxygenated horse blood of average serum and cell composition observed in our experiments.

Equations 48, 50, and 51 as approximations indicate a straight line ( $\text{BHCO}_3$ ),  $\text{pH}_s$ , curve for blood, and an approximately linear curve is observed in blood, as shown by the results of Warburg (10) and Van Slyke, Hastings, and Neill (2). However, the  $(\text{BP})_c$  value deviates slightly from the linear  $\text{pH}_s$  function assumed in Equation 52 (see Fig. 10), and the  $r$  value deviates considerably from a linear  $\text{pH}_s$  function (see Fig. 1). Both deviations tend to

make the  $(\text{BHCO}_3)_b$ , pH, curve assume towards the pH, axis a convexity that increases with increasing hemoglobin content.

In Fig. 7 are shown the  $(\text{BHCO}_3)_b$ , pH, curves for blood with  $(\text{Hb}) = 3, 6, 9$ , and  $12 \text{ mm.}$ , as calculated by Equations 45 and 46. The values of  $(\text{BP})_c$  are estimated as follows: pH, is obtained by subtracting  $-\log r$  from pH, the  $-\log r$  value being obtained from the curve of Fig. 1. With this pH, value, the  $(\text{BP})_c$  value per millimol of hemoglobin is obtained from the curve of Fig. 10. This value multiplied by  $(\text{Hb})$  gives  $(\text{BP})_c$  for the blood in question.  $(\text{BP})_c$  is calculated by Equation 54, *viz.*  $(\text{BP})_c = 0.068 (\text{P})_c (\text{pH}_c - 4.80)$ ,  $(\text{P})_c$  being estimated from Equation 17. The constant  $(\text{B})_{\text{P} + \text{HCO}_3}$  we have estimated from our data in Tables VIII to XI by the method employed in deriving Equations 15 and 16, which yields the equation

$$(53) \quad (\text{B})_{\text{P} + \text{HCO}_3} = 38 + 0.8 (\text{Hb})$$

Like the other numerical constants derived in this manner, these values of  $(\text{B})_{\text{P} + \text{HCO}_3}$  represent only the blood of the individual Mongolian pony which donated the material for these experiments. The figures are used to indicate merely the relative magnitude of the values concerned. Other normal bloods even of the same species, may differ considerably. The horse used by Van Slyke, Hastings, and Neill (2), for example, had blood with a  $(\text{P})_{\text{P} + \text{HCO}_3}$  value about 5 milli-equivalents lower than that of our animal. The levels of observed curves in samples of normal horse blood may therefore vary considerably, and the slopes somewhat, from those of the curves of Fig. 7, which are given only as illustrations of the forms that may be expected in bloods of varying hemoglobin content.

#### EXPERIMENTAL..

##### *The Alkali-Binding and Buffer Values of the Proteins of Horse Serum.*

Solutions of serum proteins were prepared by dialysis of serum. Both the serum and the water against which it was dialyzed were saturated with  $\text{CO}_2$  in order to remove the alkali from combination with the proteins, a device originated by Adolph and Ferry (26). As low a pH as can be obtained with  $\text{CO}_2$  is especially necessary to remove the alkali from combination with the serum proteins

because their isoelectric pH points are so low, about 4.7 for the albumin and 5.4 for the globulin according to Michaelis (13). The dialysis was performed in closed collodion sacs as suggested by Adair, Barcroft, and Bock (27), and was continued until the conductivity had been lowered to such a degree as to indicate an electrolyte concentration of less than 0.001 N. During the dialysis a considerable part of the globulins precipitated out. They were suspended in the solution, and the suspension was transferred to 100 cc. flasks in amounts slightly less than enough to fill the flasks. 3 cc. of standard 0.5 M  $\text{Na}_2\text{CO}_3$  and 600 mg. of NaCl were added, and a perfectly clear solution was obtained. The latter was made up to 100 cc. volume and used for determinations of the carbon dioxide-binding power at various  $\text{CO}_2$  tensions according to the technique utilized by Van Slyke, Hastings, Heidelberger, and Neill (1).

In one detail of convenience our technique differed from that utilized by them. Instead of using the gas burettes indicated in Fig. 3 of their paper, the inlet cocks were connected directly with the air and a Kipp  $\text{CO}_2$  generator, and a manometer was attached to the system. After the tonometer had been evacuated to 700 mm.,  $\text{CO}_2$  was let in until the manometer registered the desired pressure change. The desired initial  $\text{CO}_2$  tension could thus be measured directly with a sufficient degree of approximation for the present experiments.

*Calculations.*—The  $\text{H}_2\text{CO}_3$  values were calculated, for reasons discussed under "Methods of calculation" on p. 824, on the assumption that with varying protein content the  $\text{CO}_2$  solubility is proportional to the water content of the solution. Since the serum solutions were made up with approximately the same salt concentration as the original serum, the same solubility factor is used. Hence

$$\text{mm. } \text{H}_2\text{CO}_3 \text{ per liter solution} = 0.0324 \times \text{kg. H}_2\text{O per liter solution} \times p_{\text{CO}_2}$$

The pH values are calculated according to Hasselbalch's equation  
$$\text{pH} = \text{pK}' + \log \frac{\text{BHCO}_3}{\text{H}_2\text{CO}_3}$$
. The value used for  $\text{pK}'$  is 6.12. The reasons for the choice of this value, on the basis of the experimental data of Cullen (28), are given on p. 826.

Two preparations of serum proteins were used, with the results given in Tables V and VI, and in Fig. 8. From the mean of the two linear curves obtained the following formula is derived.

$$(54) \quad \text{mm. [BP]}_s = 0.068 [\text{P}]_s (\text{pH}_s - 4.80)$$

where mm. [BP]<sub>s</sub> represents milligram equivalents of alkali combined with protein, [P]<sub>s</sub> the grams of protein present.

*The Alkali-Binding and Buffer Values of the Non-Diffusible Constituents of the Blood Cells.*

The same technique employed with serum was utilized with washed cells. An unexpected difficulty was encountered, however. It was found that the cell solution, even after a fortnight's dialysis, consumes its own oxygen and produces similar amounts of CO<sub>2</sub> at such a rate as to make accurate results impossible. Within 2 hours after the cell solutions had been saturated with air plus CO<sub>2</sub> practically all of their oxygen had disappeared, the solutions had become black, and an equivalent increase in CO<sub>2</sub> had occurred. Error from production of CO<sub>2</sub> was then obviated by starting the CO<sub>2</sub> determination within 4 minutes after saturation

TABLE V.  
*Alkali-Binding Power of Dialyzed Horse Serum.*

N content of solution.....	8.05 gm. per l.
Protein content = N × 6.25.....	50.25 " " "
Water content.....	0.950 kg. per l.
Factor for calculating mm.H <sub>2</sub> CO <sub>3</sub> per l. from p <sub>CO<sub>2</sub></sub> .....	0.0308
Conductivity at 25°.....	5.8 × 10 <sup>-5</sup> mhos.
Na added as Na <sub>2</sub> CO <sub>3</sub> .....	30.53 m.-Eq. per l.
" " " NaCl.....	100.0 " " "

*Saturation Data.*

No.	CO <sub>2</sub> tension.	Total CO <sub>2</sub> .	H <sub>2</sub> CO <sub>3</sub>	BHCO <sub>3</sub>	BP	$\frac{\text{BP}}{\text{P}}$	pII
	mm.Hg	m.M. per l.	m.M. per l.	m.-Eq. per l.	m.-Eq. per l.	m.-Eq. per gm. protein	
1	16.0	21.15	0.493	20.66	9.87	0.1964	7.743
2	39.0	23.11	1.201	21.91	8.62	0.1715	7.380
3	70.2	24.80	2.163	22.63	7.90	0.1573	7.140
4	83.8	25.50	2.581	22.92	7.61	0.1515	7.068

TABLE VI.  
*Alkali-Binding Power of Dialyzed Horse Serum.*

N content of solution.....	7.55 gm. per l.
Protein content = N $\times$ 6.25.....	47.2 " " "
Water content.....	0.952 kg. per l.
Factor for calculating mm. H <sub>2</sub> CO <sub>3</sub> per l. from p <sub>CO<sub>2</sub></sub> .....	0.0309
Conductivity at 25°.....	5.8 $\times$ 10 <sup>-5</sup> mhos.
Na added as Na <sub>2</sub> CO <sub>3</sub> .....	30.60 m.-Eq. per l.
" " " NaCl.....	100.0 " " "

*Saturation Data.*

No.	CO <sub>2</sub> tension.	Total CO <sub>2</sub> .	H <sub>2</sub> CO <sub>3</sub>	BHCO <sub>3</sub>	BP	$\frac{BP}{P}$	pH
	mm. Hg	mM. per l.	mM. per l.	mM. per l.	m.-Eq. per l.	m.-Eq. per gm. protein	
1	16.9	21.60	0.522	21.08	9.52	0.2018	7.726
2	31.7	23.03	0.980	22.05	8.55	0.1812	7.472
3	59.7	24.53	1.845	22.68	7.92	0.1678	7.209
4	67.8	25.00	2.097	22.90	7.70	0.1632	7.158

TABLE VII.  
*Base Bound by Dialyzed Blood Cells.*

Total hemoglobin content (Stadie colorimetric).....	5.53 mM. per l.
Oxygen capacity.....	5.31 " " "
Oxygen capacity.....	0.960
Total hemoglobin	
H <sub>2</sub> O.....	0.9085 kilos per l.
Factor for calculating H <sub>2</sub> CO <sub>3</sub> from p <sub>CO<sub>2</sub></sub> .....	0.0295
Conductivity.....	2.3 $\times$ 10 <sup>-4</sup> mhos.
KOH added.....	30.00 mM. per l.
KCl " .....	100.0 " " "

No.	pCO <sub>2</sub>	Total CO <sub>2</sub>	Total O <sub>2</sub>	H <sub>2</sub> CO <sub>3</sub>	BHCO <sub>3</sub>	BP	pH	$\frac{dR}{dHbO_2}$	BP estimated for completely reduced solution.			BP estimated for oxy- generated solution.
									mm.-Eq. per l.	Eq. per mol Hb	Eq. per mol Hb	
1	192.6	34.22	0.04	5.68	28.54	1.46	6.820	0.47	1.44	0.260	0.73	
2	148.5	30.95	0.13	4.38	26.56	3.44	6.902	0.52	3.38	0.612	1.13	
3	106.5	28.03	0.12	3.14	24.89	5.11	7.016	0.57	5.04	0.912	1.48	
4	78.6	25.28	0.13	2.32	22.96	7.04	7.115	0.61	6.96	1.258	1.87	
5	56.8	22.76	0.03	1.68	21.08	8.92	7.218	0.63	8.90	1.608	2.24	
6	41.8	20.52	0.07	1.23	19.29	10.71	7.315	0.66	10.65	1.926	2.59	
7	29.1	18.38	0.09	0.859	17.52	12.48	7.429	0.67	12.42	2.245	2.92	
8	20.5	16.04	0.13	0.605	15.43	14.57	7.526	0.70	14.48	2.620	3.32	

was completed, and making a correction for the slight amount of  $\text{CO}_2$  formed in that time. However, the proteins themselves appear to be affected by oxidation during the saturation, and perhaps during the dialysis, and to lose some of their alkali-binding power. Some loss of oxygen-binding power (methemoglobin formation?) also occurs during dialysis. We consequently performed both dialysis and  $\text{CO}_2$  capacity determinations on reduced solutions, and made the dialysis as brief as possible. The cell solution was saturated with pure  $\text{CO}_2$  and dialyzed in narrow collodion tubes at 20°C. for 2 days against a great volume (100 liters) of distilled water, which was agitated by a stirrer.

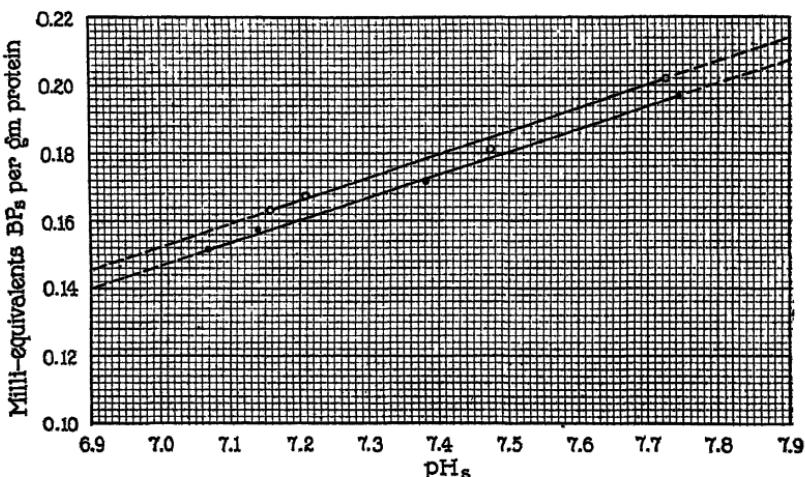


FIG. 8. Equivalents of base bound per gram of serum protein.

The water was changed every 12 hours. We attempted in this manner to attain the necessary purification with as short a dialysis as possible.

In the 2 day period we did not succeed in lowering the conductivity so far as in the longer dialyses used with the serum proteins. A choice had to be made, however, of a dialysis period such that the error due to alteration of hemoglobin should not be greater than that due to retained alkali.

The saturators were filled with mixtures of hydrogen and  $\text{CO}_2$  instead of air and  $\text{CO}_2$ . In order to make reductions as complete as possible the solution (10 cc.) was introduced into the saturator,

and the latter was evacuated, filled with hydrogen, and evacuated again. It was then rotated, so that the blood was distributed about the walls and the oxygen could be removed. The saturator was refilled with hydrogen and evacuated with a water pump three successive times to reduce the oxygen content to as nearly zero as possible, and was finally filled with the desired mixture of  $\text{CO}_2$  and  $\text{H}_2$ . The hydrogen was supplied by a Kipp generator, and was passed through pyrogallol solution and water. The deoxygenation of the solutions was almost complete. The slight amounts of oxygen left were determined and are indicated in Table VII.

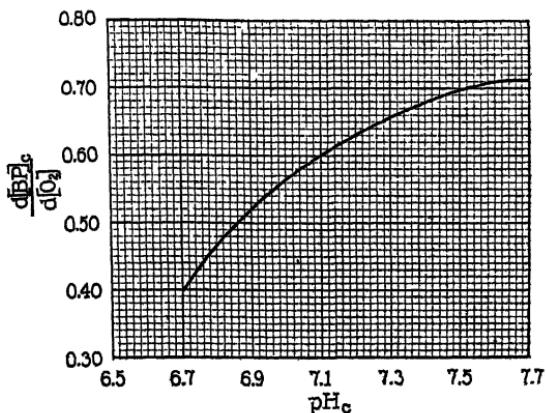


FIG. 9.

Solutions obtained in this manner did not form detectable amounts of  $\text{CO}_2$  even in 2 hours standing after they had been saturated. Oxidation effects on the proteins, or other non-diffusible, alkali-binding substances in the cell solution, appear also to have been minimized.

During the limited period in which our experiments had to be completed at Peking we succeeded in ascertaining the precautions outlined for determining the base-binding power of dialyzed cell constituents, but time then remained only for two preliminary experiments on limited volumes of solutions, one of the latter being unusually dilute. In order to avoid delay in obtaining the necessary confirmation of the results, Hastings and Harington have come to our assistance by preparing in the mean time cell solutions

by the same technique at the Hospital of The Rockefeller Institute and determining the base-binding power. The results obtained by Hastings and Harington are given in Table VII and in Fig. 10.

*Calculations.*—The  $\text{H}_2\text{CO}_3$  and pH values are calculated as described in connection with the experiments with the serum protein solutions. The figures for completely reduced cell contents are calculated as follows from those determined on the not quite entirely reduced solutions. On the basis of experiments by Hastings, Van Slyke, Neill, Heidelberger, and Harington<sup>7</sup> we have plotted in Fig. 9 the values of  $\frac{d[\text{BP}]_c}{d[\text{HbO}_2]}$ , the ratio indicating the increase in equivalents of alkali bound by the proteins per molecule of oxygen added to reduced hemoglobin. For each solution analyzed the decrease in base bound by the protein that would result from complete reduction of the hemoglobin is calculated as  $[HbO_2] \times \frac{d[\text{BP}]_c}{d[HbO_2]}$ . In these solutions the entire  $[\text{O}_2]$  is calculated as  $[\text{HbO}_2]$ , since the amount of oxygen physically dissolved at the low oxygen tensions used was negligible. The portion of hemoglobin that had lost its oxygen-binding power during dialysis is assumed in this calculation to have the base-binding power of reduced hemoglobin, in accordance with the results of the above authors.

From the  $[\text{BP}]_c$  figure for the reduced solution, that for the completely oxygenated solution is estimated by adding, to the  $[\text{BP}]_c$  value per millimol of hemoglobin found for the completely reduced solution,  $1 \times \frac{d[\text{BP}]_c}{d[\text{HbO}_2]}$ , since in this case  $\Delta [\text{HbO}_2]$  is 1, 1 molecule of oxygen being added to the reduced hemoglobin.

As a further check on our  $[\text{BP}]_c$  curve, we have, in the three of the four experiments with defibrinated blood, described below, in which complete analyses were performed, estimated the  $[\text{BP}]_c$  values as follows: We may write

$$[\text{BP}]_c = [\text{B}]_c - ([\text{BCl}]_c + [\text{BHCO}_3]_c + [\text{BX}]_c)$$

where  $[\text{BX}]_c$  indicates the concentration of alkali bound to diffusible anions other than Cl and  $\text{HCO}_3$ .  $[\text{BX}]_c$  is small, only about

<sup>7</sup> Hastings, A. B., Van Slyke, D. D., Neill, J. M., Heidelberger, M., and Harington, C. R., unpublished data.

0.1 as great as  $[BCl]$ , and we have estimated it by assuming that  $\frac{[BX]_c}{[BX]_s} = \frac{[BHCO_3]_c}{[BHCO_3]_s}$ ; hence  $[BX]_c = [BX]_s \frac{[BHCO_3]_c}{[BHCO_3]_s}$ .  $[BX]_s$  was calculated as  $[BX]_s = [B]_s - ([BCl]_s + [BHCO_3]_s + [BP]_s)$ . Such calculations involve the summation of a number of analytical errors, and cannot be expected to be very exact. Nevertheless, the results calculated as described from analyses of defibrinated blood and shown on Fig. 10 by hollow circles, solid circles, and hollow squares, follow the curve obtained from dialyzed cells plus  $KHCO_3 + KCl$  so closely that the evidence seems good that the curve represents fairly closely the amounts of alkali bound by the cell proteins in the blood.

The data from the experiment with dialyzed cells are given in Table VII, and, together with the data from the experiments with whole blood, in Fig. 10.

It will be seen that the oxygenated curve is slightly concave towards the pH axis, but it so nearly approximates a straight line that within the range covered there is little error in expressing it as such. Expressed by an approximate linear equation, the relationships may be indicated, *within the pH range of our data*, as follows:

For oxygenated blood,

$$(55) \quad [BP]_c = 3.6 [Hb]_c (\text{pH}_c - 6.6)$$

For reduced blood, representing reduced hemoglobin also as Hb,

$$(56) \quad [BP]_c = 3.35 [Hb]_c (\text{pH}_c - 6.74)$$

For partially oxygenated blood, we substitute  $[O_2]_c$  for  $[Hb]_c$  in Equation 55, and  $[Hb]_c - [O_2]_c$  for  $[Hb]_c$  in Equation 56, and add the two equations. We thus obtain for blood with varying degrees of oxygen saturation

$$(57) \quad [BP]_c = 3.35 [Hb]_c (\text{pH}_c - 6.74) + [O_2]_c (0.25 \text{ pH}_c - 1.18)$$

It is rather surprising that the buffer value 3.6, in the sense quantitatively defined by Van Slyke (29), found for oxygenated cell contents per mol of hemoglobin is so much greater than the buffer value of recrystallized hemoglobin (previously given as 2.64 (1), but really 3.0 when corrected, as shown in a forthcoming paper,

for the inactive hemoglobin present in the preparation). The difference appears, however, to be genuine; for the higher buffer value is also consistent with data we have obtained from hemolyzed blood. The cause may perhaps lie in the fact that in the former experiments the recrystallized hemoglobin was dissolved in solutions containing 30 milli-equivalents of sodium per liter, while in the present solution and in blood the ratio m.-Eq. K per kilo H<sub>2</sub>O is about 150. In part the difference may also be due to the presence of slight amounts of non-diffusible cell buffers other than hemoglobin, such as conjugated phosphates.

#### *Saturation Experiments with Blood.*

Horse blood was drawn from the jugular vein, and was at once defibrinated and saturated, in portions of 200 cc., with air plus varying amounts of CO<sub>2</sub>. The saturations were performed in the 800 cc. saturators illustrated in Fig. 3 of the first paper of this series (5). Our saturators differed slightly in form from the latter, in that, as viewed in longitudinal section, the walls at each end of ours sloped toward the cock at an angle of 60°, in order to facilitate drainage, instead of approaching it in a curve. (This improvement was introduced by Cullen.) The saturations were performed at barometric pressure, and the final CO<sub>2</sub> tension was determined by analysis in a manner described below.

Certain preliminary determinations, indicated in the tables, to ascertain some of the blood constants, were performed on a portion of the defibrinated blood which was not saturated.

The saturated portions were all centrifuged under paraffin, as detailed below. The paraffin protection from the air obviated errors from two sources, either of which would have been of sufficient magnitude to invalidate part of the results. It prevented escape of CO<sub>2</sub>, as already shown (5), and it prevented concentration of the serum by evaporation. Centrifugation was continued for about an hour at a speed of 1,500 revolutions per minute, and the cells were obtained so nearly free from serum that no visible layer of the latter separated at the top when a sample of the cells was allowed to stand over night.

The amounts of blood used were such as to obviate the necessity of using micro methods of analysis, and it was our intention further to insure the certainty of our results by obtaining all the

cell data directly from analyses performed on the cells, rather than indirectly from analysis of the serum and whole blood. We succeeded in carrying out this intention in all the determinations except that of chloride. We were not able to satisfy ourselves that the chloride method devised by Austin and Van Slyke (30)

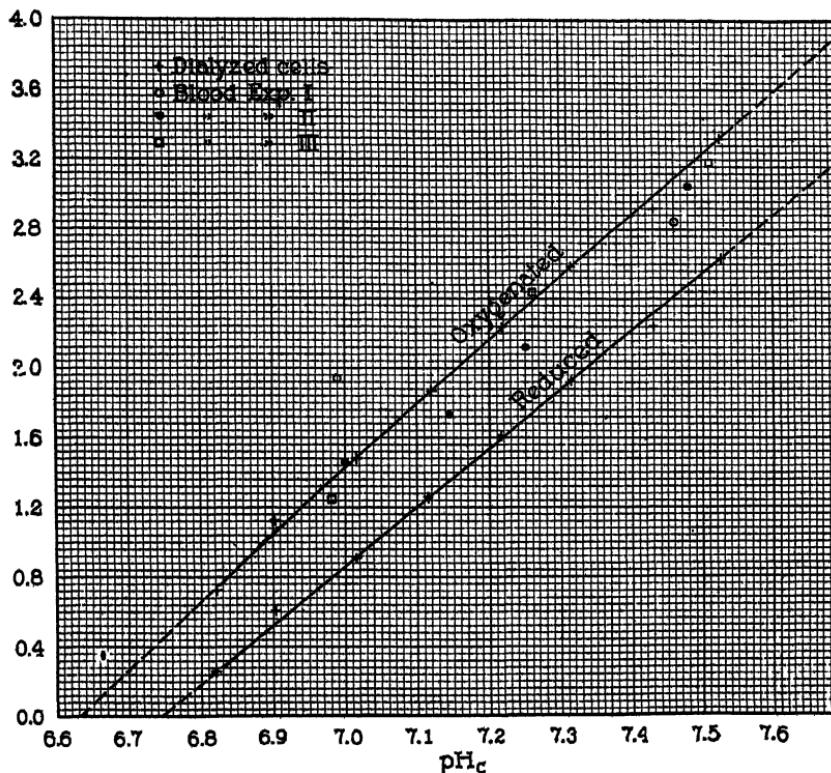


FIG. 10. Equivalents of base bound by reduced and (calculated) oxygenated, non-diffusible cell constituents, per mol of oxygen-combining capacity.

for whole blood is entirely accurate for the concentrated cells. The picric acid filtrate from the latter contains something which retards the precipitation of silver chloride, and we did not have time at our disposal to devise a new technique suited to cells. We have accordingly estimated our cell chloride content by difference from analyses of the whole blood and serum. In consequence we

cannot attribute the same degree of accuracy to the cell chloride values as to the bicarbonate values. However, by using relatively large amounts of filtrate (equivalent to 5 cc. of serum or blood) for the titrations, very consistent duplicates were obtained; and we believe the cell chloride figures indicate the changes in distribution resulting from changes in  $\text{CO}_2$  tension, although the absolute  $[\text{Cl}]_e$  values determined may parallel the true values at a distance appreciably below or above the latter.

For the determination of the water shift between serum and cells we abandoned the hematocrit method; the difficulties which it offers in measuring small changes are familiar. In its place we utilized two independent methods which served as checks on each other; *viz.*, the gravimetric determination of the water content of serum and cells, and specific gravity determination in the serum. The manner of calculating water contents from the results by each of the two procedures is indicated below in the section on "Calculations." Of the two methods it appears that the specific gravity is capable of indicating changes with the higher degree of accuracy. The gravimetric method gave fairly consistent results with cells, but in serum, where the relative water changes were smaller, it does not appear sensitive enough.

The gravities were determined in 50 cc. bottles; the difference between duplicate weighings appears not to exceed 0.5 mg., or 1 part per 100,000. Our water bath during the determinations never varied more than  $0.01^\circ$  in temperature, and usually not over  $0.003^\circ$ . The weight of water held by each bottle was redetermined immediately after each serum determination. It is probable that the ratio  $\frac{\text{weight of serum at } 38^\circ}{\text{weight of water at } 38^\circ}$  has an accuracy not much below 1 part per 100,000 and certainly not below 1 per 10,000. An error of the latter magnitude would cause one of about 1 part in 300 in estimating the water: solids ratio in serum.

In the water determinations by both methods the absolute values of  $(\text{H}_2\text{O})_s$  and  $(\text{H}_2\text{O})_c$  depend on preliminary cell volume and other determinations and are consequently subject to considerably greater error. The changes, however, due to water shift with varying  $\text{CO}_2$  tension, are determined with an accuracy dependent only on that of the direct gravimetric or specific gravity estimations.

*Saturation Technique.*—200 cc. portions of defibrinated horse blood were drawn into evacuated saturators of about 800 cc. capacity. These were filled with the desired mixture of CO<sub>2</sub> and air by the same technique employed in the other experiments, described above. To prevent development of negative pressure the saturators with high CO<sub>2</sub> tensions were rotated at room temperature for a few minutes to hasten the absorption of CO<sub>2</sub>, and more air was admitted to restore the pressure before they were placed in the bath. The saturators were then rotated in a bath at 38° until equilibrium was reached. At intervals during saturation one cock of the saturator was opened, to release the pressure. This was repeated until the internal pressure remained equal to that of the atmosphere.

The saturator was finally connected to a 250 cc. centrifuge bottle filled with paraffin oil and provided with a rubber stopper carrying two tubes as shown in Fig. 11a. With the saturator still in the bath and almost in a vertical position the blood was transferred to the centrifuge bottle by lowering the leveling bulb connected with the bottle and opening both cocks of the saturator so that air could flow in above as blood was drawn out below. Care was taken to see that the rubber and glass tubings connecting the saturator and the bottle had been completely filled with paraffin oil before the saturator cocks were opened.

When all the blood had left the saturator both cocks were closed and the rubber tubings were clamped. The saturator and the bottle were then removed from the bath and disconnected. The rubber stopper was removed from the bottle and the paraffin oil was removed as completely as possible by means of a pipette. Melted paraffin at a temperature of 50° was poured into the bottle, in sufficient amount to form a layer about 1 inch thick on top of the blood. When the paraffin had solidified the blood was centrifuged.

To remove the serum a hole about 1 cm. in diameter was bored through the paraffin by means of a heated cork borer. About 15 cc. of the serum for CO<sub>2</sub> determination were transferred over mercury to a receiver by means of a bent capillary tube,<sup>8</sup> arranged as shown in Fig. 11b. As receivers in these experiments we employed the tubes forming the lower chambers of the 2-cham-

<sup>8</sup> See Austin, Cullen, Hastings, McLean, Peters, and Van Slyke (5), p. 132.

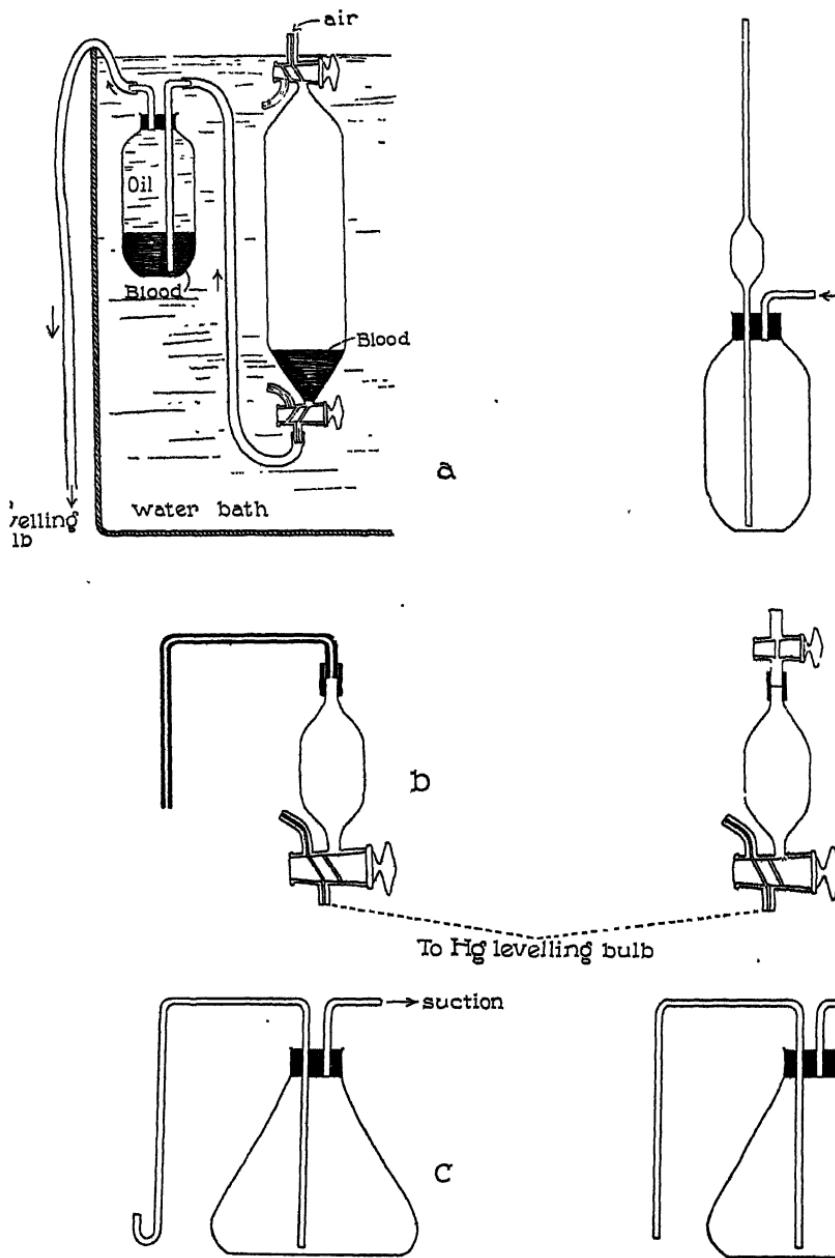


FIG. 11. Apparatus used for handling blood.

bered tonometers described in Paper I (5). Of the remainder of the serum, as much as could be withdrawn without admixture of any cells was transferred to a flask for other analyses by means of an arrangement shown in Fig. 11c.

The serum still left on the top of the cells was removed by means of a pipette as completely as possible. To take a sample of cells for CO<sub>2</sub> determination, a rubber stopper carrying a 10 cc. pipette and a bent tube (Fig. 11d) was inserted and the cells were forced up into the pipette by gentle air pressure exerted through the bent tube. The pipette was removed with the stopper, and the cells were delivered under 10 cc. of CO<sub>2</sub>-free fluoride-saponin solution, in a receiver of the kind shown in Figs. 11b and 11e. (The solution contained 0.5 per cent NaF to reduce oxidizing activity and 0.1 per cent saponin to luke the cells and yield a homogeneous solution for analysis.) The receiver plus the solution was weighed before and after the addition of the cells. Finally water was added up to a mark on the neck of the receiver at which its volume capacity had been measured. (It was slightly over 23 cc. for the tubes we used as receivers.) A mercury reservoir was then connected to the bottom cock of the tube and the top was closed by the cock shown in Fig. 11e. With the upper cock open mercury was admitted till all the air below this cock was expelled. The solution of the cells was then mixed and allowed to stand a short time before analysis.

The remainder of the cells was transferred for other analyses to a flask by means of an arrangement shown in Fig. 11f.

#### *Analytical Methods (Including a Method for Determination of the Total Base in Blood).*

The specific gravity of the serum at 38° was determined by means of 50 specific gravity bottles as previously described. The specific gravities of the whole blood and serum at room temperature (24°) were determined by means of a 10 cc. pycnometer.

The dry residue of the serum and cells was determined by drying at 120° for 18 hours.

The total CO<sub>2</sub> contents of the serum and cells were determined by means of the "constant volume" apparatus (31). The apparatus used had a total volume of 50 cc. The gas pressure was measured at 2 cc. volume. 2 cc. of the serum and 3 cc. of the cell solution prepared as described above were used for each CO<sub>2</sub>

determination. Each sample was forced into the 2 or 3 cc. pipette by pressure (see "Sampling blood," p. 131 of first paper (5)), and was delivered under a layer of water in the cup of the gas analysis apparatus. The total volume of sample plus water plus 0.5 cc. of N lactic acid was 5 cc. in each case.

The  $\text{CO}_2$  in the saturator gas was determined by Haldane's method. For calculating the  $\text{CO}_2$  tension the percentage found was multiplied by  $\frac{v}{v - 200}$  ( $v$  = volume of saturator), since the original  $v - 200$  cc. of gas mixture in equilibrium with 200 cc. of the blood was diluted by 200 cc. of air when the blood was transferred to the centrifuge bottle. Control experiments in which mercury replaced the blood showed that this procedure was accurate.

The chloride of the whole blood was determined in a preliminary sample, using the method of Austin and Van Slyke (30). The chloride of the serum was determined directly, that of the cells was calculated by difference.

The total base in the serum and in the cells was determined as follows by a method depending on the principle of Fiske's method (32) for the total base in urine.

About 50 gm. of cells or serum were weighed, and were washed into a 500 cc. flask with 350 cc. of water. In the case of the cells, 50 gm. of trichloroacetic acid, dissolved in 50 cc. of water, were added with constant shaking. The mixture was made up to 500 cc. volume and mixed. For the serum only 15 gm. of trichloroacetic acid were used.

100 cc. of the filtrate were measured into a platinum dish and 2 cc. of concentrated sulfuric acid were added. The mixture was concentrated on a water bath, then the sulfuric acid was driven off with a small flame, and finally the residue was carefully ignited until the ash was white, care being taken that all parts of the dish had been momentarily subjected to a dull red heat.

When the dish had cooled 20 cc. of water and 1 drop of methyl orange were added. The dish was gently agitated until the residue, all or nearly all, had dissolved. The solution was usually neutral. If it was acid it was titrated to neutrality with 0.1 N NaOH from a micro burette. It appears preferable to underheat the sulfates during ignition, and if necessary make a correction for the slight amount of acid left, rather than to risk overheating them and the chance of some loss of  $\text{SO}_3$ .

0.5 cc. of concentrated HCl was added. The residue (calcium phosphate) dissolved completely. The solution was rinsed into a 200 cc.

beaker with 80 cc. of water, and was heated to boiling. 5 cc. of 5 per cent barium chloride solution were added drop by drop. After standing 20 minutes the precipitate was filtered on a Gooch crucible, dried, and weighed.

The barium sulfate weighed represents the total base plus any free sulfuric acid not driven off during the ignition and minus the base bound by the total phosphoric acid present in the trichloroacetic acid filtrate. The amount of free sulfuric acid left (usually none) was given by the titration. The total phosphoric acid was determined by Brigg's modification (33) of the Bell-Doisy method.

1 molecule of phosphoric acid was taken as equal to one equivalent of base, since the phosphoric acid must be in the form of *m*-phosphate in the ignited residue. The following experiment may be cited to illustrate the method of calculation and the degree of accuracy attainable with the method.

10 cc. of m/10 KCl and 1 cc. of m/30 Na<sub>2</sub>HPO<sub>4</sub> were measured into a platinum crucible. 0.5 cc. of H<sub>2</sub>SO<sub>4</sub> was added and the mixture was evaporated to dryness and ignited. The residue was dissolved in 50 cc. of water and 1 drop of methyl orange was added. The solution was acid, and 0.78 cc. of 0.1 N NaOH was required for neutralization. The titrated solution was washed into a beaker and the sulfate was determined as described.

0.1296 gm. of BaSO<sub>4</sub> corresponding to 0.001109 equivalent of base was obtained. To obtain the total base in the original mixture 0.000033 equivalent due to the phosphate was added to it, and 0.000078 equivalent due to free sulfuric acid was subtracted from it, giving 0.001064 as against the theoretical value of 0.001067 equivalent.

The corrections due to phosphates were found to be 0.002 and 0.003 equivalents per kilo of serum and cells respectively. As these corrections were small in proportion to the total base in the serum and the cells, and varied but little, they were assumed to be constant, and the same values were used for all the determinations.

#### *Methods of Calculation.*

The following symbols are used:

mm.	= millimols.
m.-Eq.	= milli-equivalents.
G <sub>b</sub> , G <sub>s</sub> , G <sub>c</sub> ,	= specific gravities of blood, serum, and cells at 38°, water at the same temperature being 1.

$W_b$ , $W_s$ , $W_c$ ,	= weights of blood, serum, and cells in kilos.
$H_2O_b$ , $H_2O_s$ , $H_2O_c$ ,	= water of blood, serum, and cells in kilos.
$R_b$ , $R_s$ , $R_c$ ,	= dry residue of blood, serum, and cells in kilos.
$P_b$ , $P_s$ , $P_c$ ,	= protein of blood, serum, and cells in grams.
$B_b$ , $B_s$ , $B_c$ ,	= base of blood, serum, and cells in milligram equivalents.

$Cl$ ,  $CO_2$ ,  $H_2CO_3$ ,  $BHCO_3$ ,  $H^+$ , similarly to  $B$ .

$$[Cl]_s = \frac{Cl_s}{H_2O_s}, [B]_s = \frac{B_s}{H_2O_s} \dots \text{etc.}$$

$$(Cl)_s = \frac{Cl_s}{W_b}, (B)_s = \frac{B_s}{W_b}, (B)_c = \frac{B_c}{W_b} \dots \text{etc.} = \text{milli-equivalents per kilo blood.}$$

$Hb$  = hemoglobin in milligram molecules of oxygen capacity.

The manner in which our data were calculated is shown by the following formulas, which we used. Equations III to X inclusive indicate the calculation of the blood constants. The specific gravity and volume data of Equations I and II, which are used in the other calculations (except Equation VII) depend on the cell volume, and are therefore constant for a given blood only at a given  $CO_2$  and  $O_2$  tension. Consequently we have made a practice of performing the preliminary Kjeldahl, dry residue, and specific gravity determinations on the cells and serum separated in the course of the cell volume determination, as the simplest way to insure separation of cells and plasma for volume determination and for determination of the blood constants under identical conditions.

It will be noted that all concentrations are calculated as  $\frac{\text{weight of solute}}{\text{weight of water}}$  or  $\frac{\text{mols of solute}}{\text{weight of water}}$  in accordance with the basic law concerning osmotic pressure discussed under III in the introduction to this paper.

$$(I) \quad \frac{W_s}{W_b} = \frac{G_s V_s}{G_b V_b} \quad (\text{from the principle that mass} = \text{volume} \times \text{density.})$$

$\frac{V_s}{V_b}$  from centrifugation in graduated tubes.

$$(II) \quad \frac{W_c}{W_b} = 1 - \frac{W_s}{W_b}$$

$$(III) \quad \frac{R_s}{W_b} = \frac{W_s}{W_b} \times \frac{R_s}{W_s}$$

$\frac{W_s}{W_b}$  is found from I,  $\frac{R_s}{W_s}$  from dry residue determination.

$$(IV) \quad \frac{R_s}{W_b} = \frac{W_s}{W_b} \times \frac{R_s}{W_s}$$

$\frac{W_c}{W_b}$  is found from II,  $\frac{R_c}{W_c}$  from dry residue determination.

$$(V) \quad \frac{P_s}{W_b} = \frac{W_s}{W_b} \times \frac{P_s}{W_s}$$

$\frac{W_s}{W_b}$  is found from I,  $\frac{P_s}{W_s}$  by Kjeldahl on weighed serum sample.

Protein is calculated as  $N \times 6.25$ .

$$(VI) \quad \frac{P_c}{W_b} = \frac{W_c}{W_b} \times \frac{P_c}{W_s}$$

$\frac{W_c}{W_b}$  is found from II,  $\frac{P_c}{W_c}$  by Kjeldahl on weighed cell sample.

Cell protein is calculated as  $N \times 5.78$  since Kossel (34) gives 17.31 as the N content of horse hemoglobin, and  $\frac{1}{0.1731} = 5.78$ .

$$(VII) \quad \frac{Hb}{W_b} = \frac{Hb}{V_b} \times \frac{V_b}{W_b} = \frac{Hb}{V_b \times G_b}$$

$\frac{Hb}{V_b}$  is found by oxygen capacity determination.

$$(VIII) \quad (B)_s = \frac{B_s}{W_b} = \frac{W_s}{W_b} \times \frac{B_s}{W_s}$$

$\frac{W_s}{W_b}$  is found from I,  $\frac{B_s}{W_s}$  by analysis.

$$(IX) \quad (B)_c = \frac{B_c}{W_b} = \frac{W_c}{W_b} \times \frac{B_c}{W_s}$$

$\frac{W_c}{W_b}$  is found from II,  $\frac{B_c}{W_c}$  by analysis of weighed sample of cells.

$$(X) \quad f = \text{factor for calculating } \frac{H_2O_s}{W_b} \text{ from specific gravity of serum.}$$

For changes as small as those that occur in the solid content of the serum from the water exchange caused by varying  $\text{CO}_2$  and  $\text{O}_2$  tensions (migration of constituents other than water is a negligible factor), the solid content of the serum may be assumed to vary in a linear manner with  $G_s - 1$ .

$$\frac{R_s}{W_s} = f (G_s - 1)$$

Solving for  $f$  we have

$$f = \frac{R_s}{W_s} \times \frac{1}{G_s - 1}$$

$f$  being determined from  $G_s$  and  $\frac{R_s}{W_s}$  in a sample of serum separated at any  $\text{CO}_2$  tension, we may use  $f$  to determine  $(\text{H}_2\text{O})_s$  in any other serum sample from the same blood. If we substitute  $(R)_s + (\text{H}_2\text{O})_s$  for  $W_s$  in the above and solve for  $(\text{H}_2\text{O})_s$  we obtain

$$(\text{H}_2\text{O})_s = R_s \left( \frac{1}{f(G_s - 1)} - 1 \right), \text{ or } \frac{(\text{H}_2\text{O})_s}{W_b} = \frac{R_s}{W_b} \left( \frac{1}{f(G_s - 1)} - 1 \right)$$

$$(XI) \quad [\text{H}_2\text{CO}_3]_s = [\text{H}_2\text{CO}_3]_c = 0.0324 P_{\text{CO}_2}$$

(Discussion below.)

$$(XII) \quad [\text{CO}_2]_s = \frac{\text{CO}_2_s}{\text{H}_2\text{O}_s} = \frac{W_s}{\text{H}_2\text{O}_s} \times \frac{\text{CO}_2_s}{W_s} = \frac{W_s}{\text{H}_2\text{O}_s} \times \frac{\text{CO}_2_s}{V_s G_s}$$

$\frac{W_s}{\text{H}_2\text{O}_s}$  is determined on the weighed serum sample,  $\frac{\text{CO}_2_s}{V_s}$  is determined on the measured 2 cc. sample.

Similarly

$$[\text{Cl}]_s = \frac{W_s}{\text{H}_2\text{O}_s} \times \frac{\text{Cl}_s}{W_s}$$

$$[\text{B}]_s = \frac{W_s}{\text{H}_2\text{O}_s} \times \frac{\text{B}_s}{W_s}$$

and likewise for

$$[\text{CO}_2]_c, [\text{Cl}]_c, \text{ and } [\text{B}]_c$$

$$(XIII) \quad [\text{BHCO}_3]_s = [\text{CO}_2]_s - [\text{H}_2\text{CO}_3]_s$$

$$[\text{BHCO}_3]_c = [\text{CO}_2]_c - [\text{H}_2\text{CO}_3]_c$$

$$(XIV \text{ a}) \quad (\text{H}_2\text{O})_s = \frac{\text{H}_2\text{O}_s}{W_b} = \frac{\text{H}_2\text{O}_s}{R_s} \times \frac{R_s}{W_b} \text{ from gravimetric water}$$

$$(XIV \text{ b}) \quad = \frac{R_s}{W_b} \left( \frac{1}{f(G_s - 1)} - 1 \right) \text{ from specific gravity.}$$

$\frac{R_s}{W_b}$  is found from Equation III,  $f$  from Equation X.

$$(XV \text{ a}) \quad (\text{H}_2\text{O})_c = \frac{\text{H}_2\text{O}_c}{W_b} = \frac{\text{H}_2\text{O}_c}{R_c} \times \frac{R_c}{W_b} \text{ from gravimetric water}$$

$$(XV \text{ b}) \quad = \frac{\text{H}_2\text{O}_b}{W_b} - \frac{\text{H}_2\text{O}_s}{W_b} \text{ from specific gravity of serum and water content of whole blood.}$$

$\frac{R_c}{W_b}$  is found from Equation IV,  $\frac{\text{H}_2\text{O}_b}{W_b}$  from water determination on whole blood.

$$\frac{\text{H}_2\text{O}_s}{W_b} \text{ is found from XIV b.}$$

$$(XVI) \quad [\text{Hb}]_c = \frac{\text{Hb}}{\text{H}_2\text{O}_c} = \frac{\text{Hb}}{W_b} \times \frac{W_b}{\text{H}_2\text{O}_c}$$

$\frac{\text{Hb}}{W_b}$  is found from VII,  $\frac{W_b}{\text{H}_2\text{O}_c}$  from XV.

$$(XVII) \quad [\text{P}]_s = \frac{\text{P}_s}{\text{H}_2\text{O}_s} = \frac{\text{P}_s}{W_b} \times \frac{W_b}{\text{H}_2\text{O}_s}$$

$\frac{\text{P}_s}{W_b}$  is found from V,  $\frac{W_b}{\text{H}_2\text{O}_s}$  from XIV.

$$(XVIII) \quad [\text{P}]_c = \frac{\text{P}_c}{\text{H}_2\text{O}_c} = \frac{\text{P}_c}{W_b} \times \frac{W_b}{\text{H}_2\text{O}_c}$$

$\frac{\text{P}_c}{W_b}$  is found from VI,  $\frac{W_b}{\text{H}_2\text{O}_c}$  from XV.

$$(XIX) \quad [\text{H}^+] = 7.6 \times 10^{-7} \times \frac{[\text{H}_2\text{CO}_3]}{[\text{BHCO}_3]} \quad (\text{In either cells or serum. Discussion of 7.6 factor below.})$$

$$(XX) \quad \text{pH} = 6.12 + \log \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]} \quad (\text{In either cells or serum. Discussion of 6.12 factor below.})$$

$$(XXI) \quad [\text{BP}]_s = 0.068 [\text{P}]_s (\text{pH}_s - 4.80)$$

[P]<sub>s</sub> from XVII. Numerical constants from Equation 54.

$$(XXII) \quad [\text{BP}]_c = [\text{Hb}]_c \times (\text{Equivalents base bound per mol Hb.})$$

The base bound per mol was estimated graphically from Fig. 10.

[Hb]<sub>c</sub> from XVI.

*Discussion of Methods of Calculation.*—For the most part the formulas used are obvious, but the following demand some explanation.

*Equation VII. The Millimolar Concentration of Hemoglobin.*—The millimolar concentration of hemoglobin per 1,000 gm. of blood was estimated from the oxygen capacity on the assumption that 1 molecule of hemoglobin binds 1 of oxygen when saturated with atmospheric air.

The grams of hemoglobin are estimated by multiplying the millimolar oxygen capacity of the blood by 16.7. Hüfner showed that 1 molecule of oxygen or carbon monoxide combines with 16,700 gm. of ox hemoglobin, and for lack of figures determined directly on horse hemoglobin we have used this factor. The *hemoglobin weights* that we give can be considered as only approximately accurate, because of the uncertainty regarding the degree of exactness of the factor 16.7. We have, however, indicated the weights calculated by it, in order to check the relationships between hemoglobin, total cell protein, and cell solids.

*Equation XI. [H<sub>2</sub>CO<sub>3</sub>], Milligram Molecules of Free CO<sub>2</sub> per 1,000 Gm. H<sub>2</sub>O in Cells or Serum.*—[H<sub>2</sub>CO<sub>3</sub>] has been calculated on the assumption that in the water of both cells and serum CO<sub>2</sub> has the same solubility as in a salt solution with the same salt: H<sub>2</sub>O ratio, *viz.* 160 mm. of salt per 1,000 gm. of water. Geffcken (35) has found that presence of an inorganic colloid (As<sub>2</sub>S<sub>3</sub>) does not alter the solvent power of water for CO<sub>2</sub>. It appears that the proteins present in blood behave in a similar manner, affecting the solvent power of the water as little as they do its vapor tension (Neuhausen, 7).

From the data on the relative solubility of  $\text{CO}_2$  in KCl solutions (Geffcken) and in NaCl solutions (Bohr, quoted by Geffcken) the mean solubility of  $\text{CO}_2$  per gram of water in 0.16 m solutions of these salts is estimated at 98.8 per cent of its solubility in pure water.

Estimation of  $\alpha_{\text{CO}_2}$  at 38° in the blood water as 98.8 per cent of that in pure water gives  $\alpha_{\text{CO}_2} = 0.988 \times 0.555 = 0.548$ ; whence mm.  $\text{H}_2\text{CO}_3$  per liter of water in blood =  $\frac{0.548 \text{ pco}_2}{2.24 \times 760} = 0.0322 \text{ pco}_2$ . To calculate  $\text{H}_2\text{CO}_3$  per kilo of blood water, we multiply the factor 0.0322 by 1.007, the volume in liters of a kilo of water at 38°. Hence  $[\text{H}_2\text{CO}_3] = 0.0324 \text{ pco}_2$ .

If, on the basis of the above assumption that the blood colloids do not significantly alter the solubility of  $\text{CO}_2$  in the blood water, we calculate the *relative* solubility coefficient (the solubility per unit volume of solution compared with the solubility per unit volume of water) for  $\text{CO}_2$  in the total serum we obtain the value  $0.988 \times 0.92 \times 1.027 = 0.935$ . (0.988 = relative solubility in serum water; 0.92 = gm. of water per gm. of serum; 1.027 = specific gravity of serum.)

Similarly, assuming that the cells contain 65 per cent of water and have a specific gravity of 1.10, we calculate that the relative  $\text{CO}_2$  solubility coefficient is  $0.988 \times 0.65 \times 1.10 = 0.71$ .

Bohr (36) found that oxygen had a relative solubility of 0.975 in serum, and assumed the same relative solubility for  $\text{CO}_2$ . For blood cells Bohr estimated indirectly, from hydrogen solubilities in whole blood, a relative  $\text{CO}_2$  solubility of 0.81. Both of Bohr's coefficients are higher than those estimated by us from the  $\text{H}_2\text{O}$  and salt content of serum and cells.

However, the principle of parallel gas solubilities used by Bohr was shown by Geffcken in 1904 (35) to be inexact. "Die Grösse der relativen Löslichkeitsverniedrigung ist sehr abhängig von der Nature des Gases." Bohr's own data on the solubility ratios of  $\text{O}_2$  and  $\text{N}_2$  in various water solutions show considerable variation. The accuracy with which Bohr's  $\text{O}_2$  and  $\text{H}_2$  relative solubilities can be translated into  $\text{CO}_2$  relative solubilities is according to both Geffcken's results and his own, uncertain.

Some preliminary solubility determinations performed directly with  $\text{CO}_2$  itself in serum and cell contents acidified to a pH suffi-

ciently low to prevent the combination of any  $\text{CO}_2$  with bases have given results more in accord with the coefficients we have estimated from the water content than with those Bohr has estimated from  $\text{H}_2$  and  $\text{O}_2$  solubilities. The use of a  $\text{CO}_2$  solubility factor proportional to the water content also corrects automatically for variations in the solid content of the cells and serum. While minor factors may be found to alter the  $\text{CO}_2$  solubilities calculated as proportional to the water content, the solubilities so calculated appear sufficiently close to serve our present purposes; e.g., the difference between the  $\text{pH}_s$  calculated by our solubility coefficient and the  $\text{pH}_s$  calculated by Bohr's is only 0.02, and the correct coefficient, if it is not exactly the one we use, appears to be nearer to it than to Bohr's. The  $\text{pH}_s$  values calculated by Equation XX may, therefore, be assumed to be subject to less than 0.01 pH error from possible inaccuracy in  $\alpha_{\text{CO}_2}$ .

*Equation XIX.*  $[\text{H}^+]$ , Gram Equivalent of Ionized Hydrogen per 1,000 Gm. of Water.—The value of the  $K'$  of Henderson's equation  $[\text{H}^+] = K' \frac{[\text{H}_2\text{CO}_3]}{[\text{BHCO}_3]}$ , we have calculated for serum from the results of Cullen (28). We have calculated  $[\text{H}_2\text{CO}_3]$  and  $[\text{BHCO}_3]$  as in Equations XI and XIII above, and estimated  $\text{pK}'$ , the negative logarithm of  $K'$ , as  $\text{pK}' = \text{pH} - \log \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]}$ .  $[\text{CO}_2]$  has been estimated as mm.  $\text{CO}_2$  per liter  $\times 1.06$  ( $1.06 = \frac{1}{1.026 \times 0.92}$ , 1.026 being taken as the average specific gravity of horse serum, 0.92 as the water content). Cullen's data yield for both serum and plasma of horse blood the average  $\text{pK}'$  value of 6.12, the maximum variation (except in one determination out of twenty-three) being from 6.09 to 6.14.

We have therefore used for the constant  $K'$ , the value  $7.58 \times 10^{-7}$ , of which 6.12 is the negative logarithm.

Warburg (10) has shown that the  $\text{pK}'$  value is a function of the concentration of cations,  $\text{pK}' = \text{pK}_1 - 0.46 \sqrt[3]{[\text{B}]}$ ,  $\text{pK}_1$  being the value of  $\text{pK}'$  in bicarbonate solutions at infinite dilution.  $[\text{B}]_s$  and  $[\text{B}]_c$  in blood are so near together, that the extreme difference observed in our experiments would cause  $\text{pK}'_s$  to differ from  $\text{pK}'_c$  by only about 0.01. We have therefore used the same  $K'$  and  $\text{pK}'$  values for cells as for serum.

TABLE VIII *a.*  
*Experiment 1.*  
*Preliminary Determinations for Calculation of Blood Constants.*

Determination.	Symbol.	Unit.	Blood.	Serum.	Cell.
Oxygen capacity per l.	Hb:V <sub>b</sub>	mm.	5.69	0.710	0.290
Volume per l.	V <sub>b</sub> :V <sub>e</sub> ; V <sub>e</sub> :V <sub>b</sub>	l.	0.8357	0.9188	0.6390
Water per gm.	H <sub>2</sub> O <sub>b</sub> :W <sub>b</sub> , H <sub>2</sub> O <sub>e</sub> :W <sub>b</sub> , H <sub>2</sub> O <sub>e</sub> :W <sub>b</sub>	gm.	0.1643	0.0812	0.3610
Solids per gm.	R <sub>b</sub> :W <sub>b</sub> , R <sub>e</sub> :W <sub>e</sub> , R <sub>e</sub> :W <sub>b</sub>	"	"	0.0118	0.0579
Nitrogen per gm.	N <sub>b</sub> :W <sub>b</sub> , N <sub>e</sub> :W <sub>e</sub>	"	"	0.0738*	0.3342†
Protein per gm.	P <sub>b</sub> :W <sub>b</sub> , P <sub>e</sub> :W <sub>e</sub>	m. <sup>-</sup> Eq.	82.7		
Chloride per kilo.	Cl <sub>b</sub> :W <sub>b</sub>	"			
Base per kilo.	B <sub>b</sub> :W <sub>b</sub> , B <sub>e</sub> :W <sub>e</sub>				
Specific gravity 24°	G <sub>b</sub> , G <sub>e</sub>		1.0457	1.0257	
Weight per gm. blood	W <sub>b</sub> , W <sub>e</sub> :W <sub>b</sub>	gm.	0.697	0.363	

\* N X 6.25.

† N X 5.78.

TABLE VIII b.  
Experiment 1.  
*Calculated Constants.*

Constants.	Symbols.	Unit.
Hemoglobin per kilo blood.....	(Hb)	mM.
" " gm. "	"	gm.
Total solids " " "	(R) <sub>b</sub> = R <sub>b</sub> :W <sub>b</sub>	"
Serum " " "	(R) <sub>s</sub> = R <sub>s</sub> :W <sub>b</sub>	"
Cell " " "	(R) <sub>c</sub> = R <sub>c</sub> :W <sub>b</sub>	"
Serum protein " " "	(P) <sub>s</sub> = P <sub>s</sub> :W <sub>b</sub>	"
Cell " " "	(P) <sub>c</sub> = P <sub>c</sub> :W <sub>b</sub>	"
Serum base " kilo "	(B) <sub>b</sub> = B <sub>b</sub> :W <sub>b</sub>	mM.
Cell " " "	(B) <sub>c</sub> = B <sub>c</sub> :W <sub>b</sub>	"
Factor for calculated H <sub>2</sub> O <sub>s</sub> :W <sub>b</sub> from G <sub>s</sub> $\frac{38^{\circ}}{38^{\circ}}$ .....	f	3.09*

\* From Table VIII c.

TABLE VIII C.  
Experiment I.  
*Determinations after Saturations at 38°.*

Determination.	Symbol.	Unit.	Blood 1.		Blood 2.	
			Serum.	Cells.	Serum.	Cells.
CO <sub>2</sub> tension.....	psa	mm. Hg	19.3	19.3	106.7	106.7
CO <sub>2</sub> per kilo.....	CO <sub>2</sub> :W <sub>s</sub> , CO <sub>2</sub> :W <sub>e</sub>	mm.	20.38	9.29	34.18	19.53
Cl.....	Cl:W <sub>s</sub> , Cl:W <sub>e</sub>	m.-Eq.	100.2	41.8	95.9	53.0
Cl " "	"	"	145.3	109.2	148.1	108.6
Base " "	B <sub>s</sub> :W <sub>s</sub> , B <sub>e</sub> :W <sub>e</sub>					
Water per gm.....	H <sub>2</sub> O <sub>s</sub> :W <sub>s</sub> , H <sub>2</sub> O <sub>e</sub> :W <sub>e</sub>	gm.	0.9186	0.6508	0.9190	0.6665
Solids " "	R <sub>s</sub> :W <sub>s</sub> , R <sub>e</sub> :W <sub>e</sub>	"	0.0814	0.3494	0.0810	0.3335
Ratio, water : solids.....	H <sub>2</sub> O <sub>s</sub> :R <sub>s</sub> , H <sub>2</sub> O <sub>e</sub> :R <sub>e</sub>					
Specific gravity $\frac{38}{38}$ .....	G <sub>s</sub>		11.28	1.864	11.35	1.998
				1.02595		1.02660

TABLE VIII d.  
Experiment 1.  
*Values Calculated from Determinations on Blood Saturated at 38°.*

Relationship demonstrated.	Values calculated.	Symbol and equation.	Unit.	Blood 1.		Blood 2.	
				Serum.	Cells.	Serum.	Cells.
	$\text{CO}_2$ , per kg. water.	$[\text{CO}_2]$	mm.	22.16	14.27	37.18	29.30
	$\text{H}_2\text{CO}_3$ , " "	$[\text{H}_2\text{CO}_3]$	"	0.625	0.625	3.46	3.46
	$\text{BHCO}_3$ , " "	$[\text{BHCO}_3]$	m.-Eq.	21.52	13.64	33.72	25.84
	Cl	[Cl]	"	109.1	64.2	104.3	79.5
	Base	[B]	"	158.1	167.6	161.1	162.8
	" " " blood.	(B)	"	99.4	88.5	100.2	34.8
	Hemoglobin per kg. water.	[Hb]	mm.	27.0	27.0	25.4	25.4
	Protein per kg. water.	[P]	gm.	79.5	487	80.9	463
	pH	pH	-log [H <sup>+</sup> ]	7.658	7.459	7.109	6.993
	Base bound by protein per kg. water.	[BP]	m.-Eq.	15.7	83.5	12.7	35.1
Equality of ratio ions + mols in water serum and cells. III	Total osmolar concentration - X. Observed.	$\Sigma [M] - X = [B] + [\text{Cl}] + [\text{HCO}_3] + [\text{Hb}]$ , Equation 5.	mm.	289	272	289	294
	Total osmolar concentration. Calculated.	$\Sigma [M] = 2[B] - [\text{BP}] + [\text{Hb}]$ , Equation 7.	"	300	279	310	316

Water distribution. III	Water per kg. blood. (H <sub>2</sub> O)		kg.	0.214
	Observed gravimetric. Water per kg. blood.	"	"	0.200
	Observed sp. gr.			0.629
	Water per kg. blood. Calculated.	" by Equations 23 and 24. $\Delta(H_2O)$	"	0.639
	Water shift from Blood 1. Observed gravimetric.	"	"	0.197
	Water shift from Blood 1. Observed sp. gr.	"	"	0.643
	Water shift from Blood 1. Calculated.	" by Equations 23 and 24.	"	0.193
	Distribution ratio. Observed. I, II, III	[Cl] <sub>e</sub> : [Cl] <sub>i</sub> [HCO <sub>3</sub> ] <sub>e</sub> : [HCO <sub>3</sub> ] <sub>i</sub> = [H <sup>+</sup> ] <sub>e</sub> : " by Equation 10.	Ratio.	0.622
	Distribution ratio. Calculated.	" " 14.	"	0.667
				0.853









TABLE X a.  
*Experiment 8.*  
*Preliminary Determinations for Calculation of Blood Constants.*

Determination.	Symbol.	Unit.	Blood.	Serum.	Cell.
Oxygen capacity per l.....	Hb:V <sub>b</sub>	mM.	6.62	0.6775	0.3225
Volume per l.....	V <sub>e</sub> :V <sub>b</sub> , V <sub>c</sub> :V <sub>b</sub>	l.			
Water per gm.....	H <sub>2</sub> O <sub>b</sub> ; W <sub>b</sub> , H <sub>2</sub> O <sub>e</sub> ; W <sub>b</sub> , H <sub>2</sub> O <sub>c</sub> ; W <sub>b</sub>	gm.	0.8206	0.9143	0.6300
Solids per gm.....	R <sub>b</sub> ; W <sub>b</sub> , R <sub>e</sub> ; W <sub>e</sub> , R <sub>c</sub> ; W <sub>c</sub>	"	0.1794	0.0857	0.3700
Nitrogen per gm.....	N <sub>b</sub> ; W <sub>b</sub> , N <sub>c</sub> ; W <sub>c</sub>	"		0.01153	0.05715
Protein per gm.....	P <sub>b</sub> ; W <sub>b</sub> , P <sub>c</sub> ; W <sub>c</sub>	"		0.0721*	0.3302†
Chloride per kilo.....	Cl <sub>b</sub> ; W <sub>b</sub>	m.-Eq.	80.7		
Base per kilo.....	B <sub>b</sub> ; W <sub>b</sub> , B <sub>c</sub> ; W <sub>c</sub>	"			
Specific gravity $\frac{24}{24}$ .....	G <sub>b</sub> , G <sub>c</sub>		1.051	1.027	
Weight per kilo.....	W <sub>b</sub> :W <sub>b</sub> = G <sub>b</sub> .V <sub>b</sub> , G <sub>c</sub> .V <sub>c</sub> , W <sub>c</sub> :W <sub>b</sub> = 1-W <sub>b</sub> :W <sub>b</sub>			0.662	0.338
Specific gravity $\frac{38}{38}$ .....	G <sub>b</sub>				1.027332

\* N X 6.25.

† N X 5.78.

Hemoglobin	per kilo blood...		(Hb)		mm.	mm.
" " gm.	" "	" "	(R) <sub>b</sub> = R <sub>b</sub> ; W <sub>b</sub>		6.30	6.30
Total solids	per " gm.	" "	(R) <sub>s</sub> = R <sub>s</sub> ; W <sub>s</sub>		0.1052	0.1052
Serum " "	" "	" "	(R) <sub>e</sub> = R <sub>e</sub> ; W <sub>e</sub>		0.1794	0.1794
Cell " "	" "	" "	(P) <sub>s</sub> = P <sub>s</sub> ; W <sub>s</sub>		0.0567	0.0567
Serum protein " "	" "	" "	(P) <sub>e</sub> = P <sub>e</sub> ; W <sub>e</sub>		0.1252	0.1252
Cell " "	" "	" "	(P) <sub>s</sub> = P <sub>s</sub> ; W <sub>s</sub>		0.0477	0.0477
Serum base " kilo	" "	" "	(P) <sub>e</sub> = P <sub>e</sub> ; W <sub>e</sub>		0.1116	0.1116
Cell " " " "	" "	" "	(B) <sub>s</sub> = B <sub>s</sub> ; W <sub>s</sub>	m.-Eq.	97.7	97.7
Serum " " " "	" "	" "	(B) <sub>e</sub> = B <sub>e</sub> ; W <sub>e</sub>	"	38.9	38.9
Factor for calculating H <sub>2</sub> O <sub>s</sub> ; W <sub>s</sub> from G <sub>b</sub> 38° / 38°	f		f		3.135	

TABLE X c.  
Experiment 8.  
*Determinations after Saturation at 38°.*

Determination.	Symbol.	Unit.	Blood 1.		Blood 2.		Blood 3.	
			Serum.	Cells.	Serum.	Cells.	Serum.	Cells.
CO <sub>2</sub> tension.....	P <sub>CO<sub>2</sub></sub>	mm. Hg	14.52	14.52	40.9	40.9	117.2	117.2
CO <sub>2</sub> per kilo.....	CO <sub>2s</sub> ; W <sub>s</sub> , CO <sub>2e</sub> ; W <sub>e</sub>	mm.	18.48	7.69	25.12	12.55	35.02	20.50
Cl " " "	Cl <sub>s</sub> ; W <sub>s</sub> , Cl <sub>e</sub> ; W <sub>e</sub>	mm.	98.60	95.6			92.6	
Base " " "	B <sub>s</sub> ; W <sub>s</sub> , B <sub>e</sub> ; W <sub>e</sub>	m.-Eq.	144.7	113.3	148.4	111.5	163.2	104.8
Water " gm.....	H <sub>2</sub> O; W <sub>s</sub> , H <sub>2</sub> O; W <sub>e</sub>	gm.	0.9158	0.6350	0.9136	0.6415	0.9137	0.6514
Solids " " "	R <sub>s</sub> ; W <sub>s</sub> , R <sub>e</sub> ; W <sub>e</sub>	"	0.0842	0.3850	0.0864	0.3585	0.863	0.3486
Ratio, water : solids.....	H <sub>2</sub> O; R <sub>s</sub> , H <sub>2</sub> O; R <sub>e</sub>	"	10.87	10.740	10.60	1.790	10.58	1.817
Specific gravity 38°.....	G <sub>b</sub>			1.027256		1.027889		1.028023

TABLE X d-  
Values Calculated from Determi

Relationship demonstrated.	Values calculated.	Symbol and equation.
	$\text{CO}_2$ per kg. water. $\text{H}_2\text{CO}_3$ " " " $\text{BHCO}_3$ " " " $\text{Cl}$ " " " $\text{Base}$ " " " " " " blood. $\text{Hemoglobin}$ per kg. water. $\text{Protein}$ " " " $\text{pH}$ $\text{Base bound by protein}$ per kg. water.	$[\text{CO}_2]$ $[\text{H}_2\text{CO}_3]$ $[\text{BHCO}_3]$ $[\text{Cl}]$ $[\text{B}]$ $(\text{B})$ $[\text{Hb}]$ $[\text{P}]$ $\text{pH}$ $[\text{BP}]$
Equality of ratio ions + mols in water serum and cells. III	Total osmolar concentration - X. Observed. Total osmolar concentration. Calculated.	$\Sigma [M] - X = [B] + [\text{Cl}] + [\text{HCO}_3] + [\text{Hb}], \text{Equation 5.}$ $\Sigma [M] = 2[B] - [\text{BP}] + [\text{Hb}], \text{Equation 7.}$
Water distribution. III	Water per kg. blood. Observed gravimetric. Water per kg. blood. Observed sp. gr. Water per kg. blood. Calculated. Water shift from Blood 1. Observed gravimetric. Water shift from Blood 1. Observed sp. gr. Water shift from Blood 1. Calculated.	$(\text{H}_2\text{O})$ " " " by Equations 23 and 24. $\Delta (\text{H}_2\text{O})$ " " " by Equations 23 and 24.
Diffusible ion distribution. I, II, III	Distribution ratio. Observed. Distribution ratio. Calculated.	$[\text{Cl}]_c : [\text{Cl}]_s$ $[\text{HCO}_3]_c : [\text{HCO}_3]_s = [\text{H}^+]_s : [\text{H}^+]_c$ $r$ by Equation 10. " " " 14.

*Saturated at 38°.*

Blood 1.		Blood 2.		Blood 3.	
Serum.	Cells.	Serum.	Cells.	Serum.	Cells.
20.36	12.11	27.50	19.56	38.34	31.48
0.470	0.470	1.325	1.325	3.80	3.80
19.89	11.64	26.17	18.13	34.54	27.68
107.6	66.3	105.7	76.3	101.3	84.8
158.0	178.4	162.5	173.8	167.7	162.2
97.6	38.9	97.8	39.0	100.6	36.5
	28.9		28.1		27.7
77.4	521	79.2	497	79.4	491
7.747	7.513	7.416	7.257	7.078	6.982
15.7	94.9	14.1	66.9	12.3	37.4
<hr/>					
286	285	294	296	303	302
300	291	311	309	323	315
<hr/>					
0.616	0.218	0.602	0.224	0.600	0.228
0.608	0.213	0.592	0.229	0.589	0.232
0.612	0.209	0.600	0.221	0.586	0.235
0.000	0.000	0.014	0.006	0.016	0.010
0.000	0.000	0.016	0.016	0.019	0.019
0.000	0.000	0.012	0.012	0.026	0.026
<hr/>					
0.616		0.722		0.837	
0.586		0.693		0.802	
0.620		0.727		0.830	
0.629		0.740		0.845	

TABLE XI a.  
Experiment 4.  
*Preliminary Determinations for Calculation of Blood Constants.*

Determination.	Symbol.	Unit.	Blood.	Serum.	Cell.
Oxygen capacity per l.....	Hb:V <sub>b</sub>	mm.	6.91	0.663	0.337
Volume per l.....	V <sub>b</sub> :V <sub>e</sub> ; V <sub>e</sub> :V <sub>b</sub>	l.	0.8170	0.9103	
Water per gm.....	H <sub>2</sub> O:W <sub>b</sub> , H <sub>2</sub> O:W <sub>e</sub> , H <sub>2</sub> O:W <sub>s</sub>	gm.	0.1830	0.0897	
Solids per gm.....	R <sub>b</sub> :W <sub>b</sub> , R <sub>e</sub> :W <sub>e</sub> , R <sub>s</sub> :W <sub>s</sub>	"	"	0.01126	0.0538
Nitrogen per gm.....	N <sub>b</sub> :W <sub>b</sub> , N <sub>e</sub> :W <sub>e</sub>	"	"	0.0704*	0.312†
Protein per gm.....	P <sub>b</sub> :W <sub>b</sub> , P <sub>e</sub> :W <sub>e</sub>	m.-Eq.	77.4	148.8	120.0
Chloride per kilo.....	Cl <sub>b</sub> :W <sub>b</sub>	"			
Base per kilo.....	B <sub>b</sub> :W <sub>b</sub> , B <sub>e</sub> :W <sub>e</sub>				
Specific gravity $\frac{24^{\circ}}{24^{\circ}}$ .....	G <sub>b</sub> , G <sub>e</sub>		1.052	1.027	
Weight per kilo.....	W <sub>b</sub> :W <sub>s</sub> = G <sub>b</sub> V <sub>b</sub> :G <sub>e</sub> V <sub>e</sub> , W <sub>e</sub> :W <sub>s</sub> = 1-W <sub>e</sub> :W <sub>s</sub>			0.647	0.353
Specific gravity $\frac{38^{\circ}}{38^{\circ}}$ .....	G <sub>s</sub>				1.028353

\* N X 6.25.

† N X 5.78.

TABLE XI b.  
*Experiment 4.*  
*Calculated Constants.*

Constants.		Symbols.		Unit.	
	(Hb)			m.m.	
Hemoglobin	per kilo blood.....			6.57	
" " gm.	" .....			0.1096	
Total solids	" "			0.1830	
Serum	" "	$(R)_s = R_s : W_s$			
Cell	" "	$(R)_c = R_c : W_s$		0.05804	
Serum protein	" "	$(R)_p = R_p : W_s$		"	
Cell	" "	$(P)_c = P_c : W_b$		0.1250	
Serum base	kilo	$(P)_s = P_s : W_b$		0.0455	
Cell	" "	$(B)_c = B_c : W_b$		0.1102	
		$(B)_s = B_s : W_b$		96.3	
		$(B)_p = B_p : W_b$		"	
Factor for calculating $H_2O_2 : W_s$ from $G_s$	$\frac{38}{28}$	$f$		(39.4)*	
				3.165	

\* Calculated as 6(Hb). Direct determination lost.

TABLE XI c.  
*Experiment 4.*  
*Determinations after Saturation at 38°.*

Determination.	Symbol.	Unit.	Blood 1.		Blood 2.		Blood 3.	
			Serum.	Cells.	Serum.	Cells.	Serum.	Cells.
$CO_2$ tension.....	$p_{CO_2}$	mm. Hg	19.6	19.6	52.9	52.9	127.3	127.3
$CO_2$ per kilo.....	$CO_{2e} : W_s$	mm.	21.28	9.13	28.23	15.00	35.90	21.95
Cl	" "	m.-Eq.	96.7	94.7			91.6	
Specific gravity $\frac{38}{38}$ .....	$G_s$		1.027943				1.028774	

TABLE XI d—  
Values Calculated from Determinations

Relationship demonstrated.	Values calculated.	Symbol and equation.
	CO <sub>2</sub> per kg. water. H <sub>2</sub> CO <sub>3</sub> " " " BHCO <sub>3</sub> " " " Cl " " " Base " " " Hemoglobin per kg. water. Protein " " " pH Base bound by protein per kg. water.	[CO <sub>2</sub> ] [H <sub>2</sub> CO <sub>3</sub> ] [BHCO <sub>3</sub> ] [Cl] [B] [Hb] [P] pH [BP]
Equality of ratio ions + mols in water serum and cells. III	Total osmolar concentration — X. Observed. Total osmolar concentration. Calculated.	$\Sigma [M] - X = [B] + [Cl] + [HCO_3] + [Hb]$ , Equation 5. $\Sigma [M] = 2[B] - [BP] + [Hb]$ , Equation 7.
Water distribution. III	Water per kg. blood. Observed gravimetric. Water per kg. blood. Observed sp. gr. Water per kg. blood. Calculated. Water shift from Blood 1. Observed gravimetric. Water shift from Blood 1. Observed sp. gr. Water shift from Blood 1. Calculated.	(H <sub>2</sub> O) " " " by Equations 23 and 24. $\Delta (H_2O)$ " " " by Equations 23 and 24.
Diffusible ion distribution. I, II, III	Distribution ratio. Observed. Distribution ratio. Calculated.	[Cl] <sub>c</sub> : [Cl] <sub>e</sub> [HCO <sub>3</sub> ] <sub>c</sub> : [HCO <sub>3</sub> ] <sub>e</sub> = [H <sup>+</sup> ] <sub>c</sub> : [H <sup>+</sup> ] <sub>e</sub> r by Equation 10.

*Explanation of Tables VIII, IX, X, and XI.*—The abbreviations used have already been defined on pp. 819-820.

The data from each experiment are given in four sections headed *a*, *b*, *c*, and *d*, of the corresponding table. The calculations involved have already been outlined.

In the final section (headed *d*), the Roman numerals, I, II, III, used at the left of the pages, indicate the laws of solutions involved in the blood relationships demonstrated, the laws being referred to by the numeral

## Experiment 4.

Variations on Blood Saturated at 38°.

Unit.	Blood 1.		Blood 2.		Blood 3.	
	Serum.	Cells.	Serum.	Cells.	Serum.	Cells.
mm.	23.35	14.35	31.00	22.91	39.55	33.50
"	0.635	0.635	1.715	1.715	4.122	4.122
"	22.71	13.61	29.28	21.19	35.43	29.38
m.-Eq.	106.3	63.5	104.1	70.7	100.8	79.8
"	161.7	(181.5)	163.9	(173.6)	166.3	(165.6)
mm.		30.0		28.7		27.6
gm.	76.1	571	77.4	528	78.6	525
-log [H <sup>+</sup> ]	7.686	7.450	7.352	7.212	7.055	6.972
m.-Eq.	15.0	90.8	13.4	63.7	12.1	36.2
mm.	290	289	297	294	303	302
"	308	302	314	312	321	323
kg.	—	—	—	—	—	—
"	0.598	0.219	—	—	0.579	0.238
"	0.602	0.215	0.591	0.226	0.579	0.238
"	—	—	—	—	—	—
"	0.000	0.000	—	—	0.019	0.019
"	0.000	0.000	0.011	0.011	0.023	0.023
Ratio.		0.597		0.679		0.792
"		0.597		0.723		0.830
"		0.639		0.738		0.832

headings under which they are respectively discussed in the theoretical introduction of the paper.

In the tabulation of data showing the osmolar relationships of cells and serum the symbol X is used to indicate the small portion of non-protein anions other than Cl<sup>-</sup> and HCO<sub>3</sub><sup>-</sup>; i.e.,

$$\Sigma [M]_s = [B]_s + [Cl]_s + [HCO_3]_s + [X]_s, \text{ hence}$$

$$[B]_s + [Cl]_s + [HCO_3]_s = \Sigma [M]_s - [X]_s$$

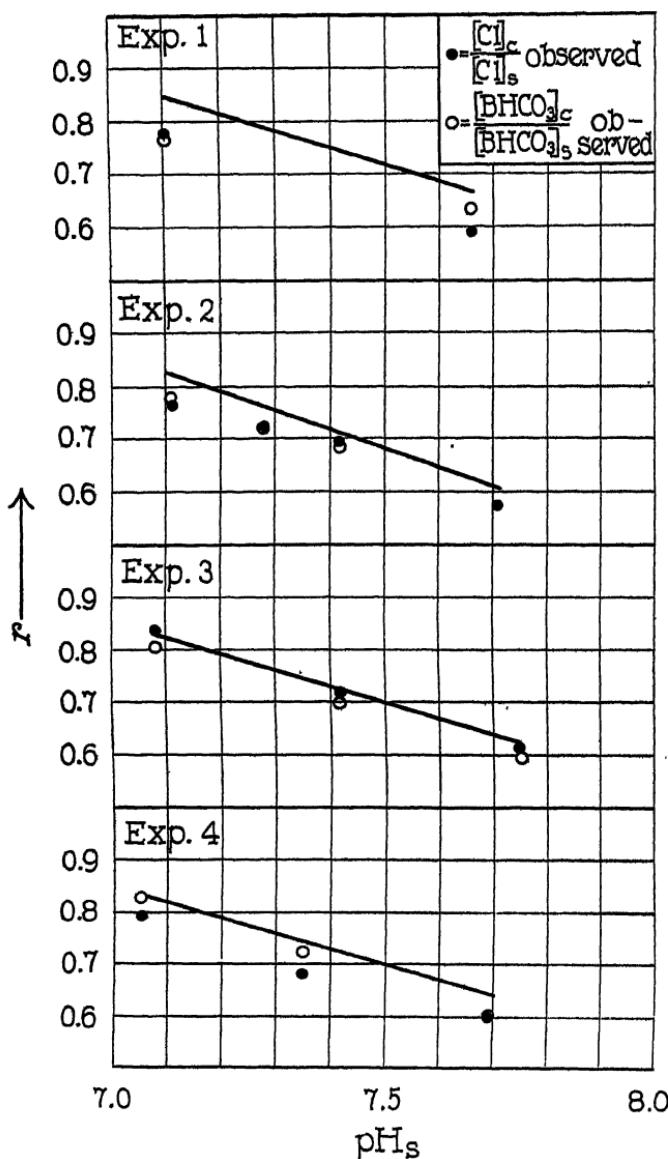


FIG. 12. Comparison of observed  $[\text{Cl}']_{\text{o}} : [\text{Cl}']_{\text{s}}$  and  $[\text{BHCO}_3']_{\text{o}} : [\text{BHCO}_3']_{\text{s}}$  values with values calculated by Equation 10 from base-binding powers of cell and serum proteins. Calculated values are indicated by the curves.

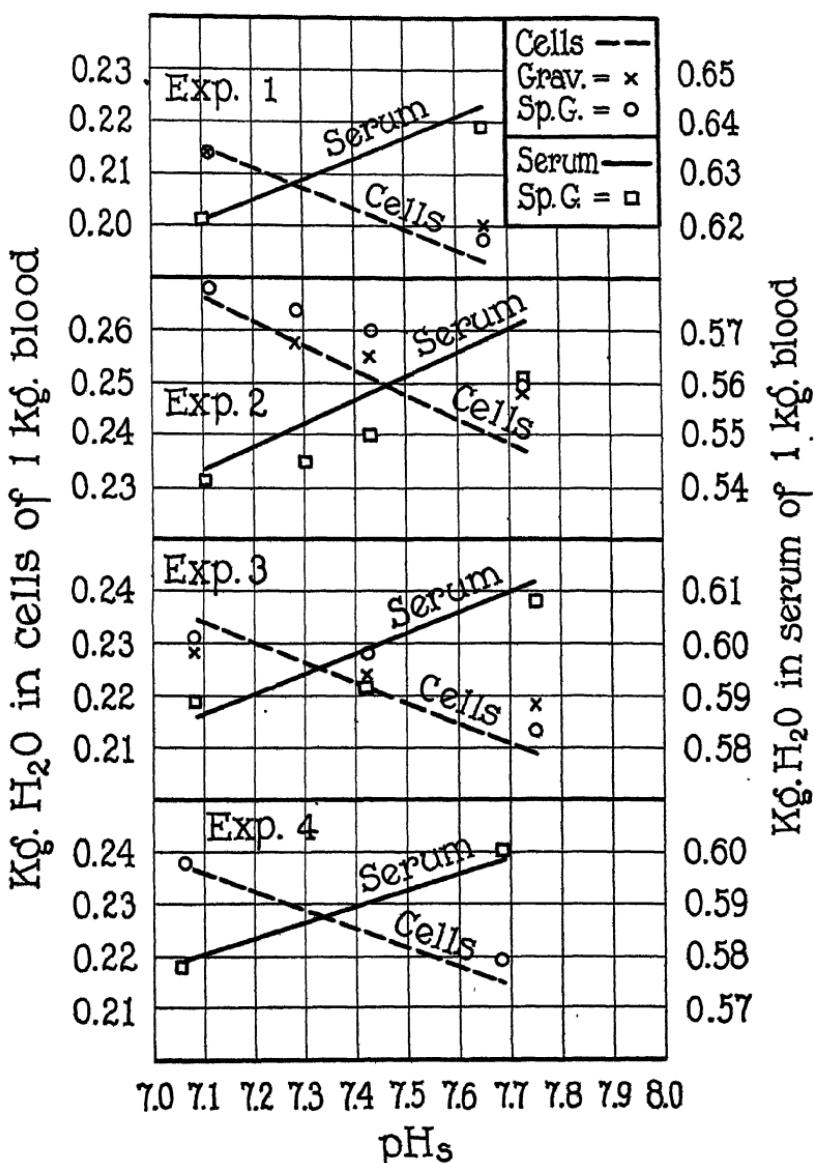


FIG. 13. Comparison of observed and calculated water distribution. Cell and serum water contents calculated by Equations 23 and 24 are indicated by the curves. Water contents observed by the gravimetric and specific gravity methods are indicated by the marked points.

Warburg's data from  $\text{NaHCO}_3 + \text{NaCl}$  solutions indicate for  $[B] = 0.166$  to  $0.170 \text{ N}$ , values for  $pK'$  of 6.11, 6.08, and 6.11 respectively when reduced from Bjerrum's pH unit to Sörensen's by subtracting 0.05.<sup>9</sup> The agreement with the 6.12 value obtained as the mean of Cullen's results is satisfactory.

We have continued to use the Sörensen pH unit ( $0.1 \text{ N HCl}$  being assumed to have a pH of 1.090 at  $38^\circ$ ) rather than the Bjerrum unit, because practically all biochemical data except Warburg's are at present in terms of the Sörensen unit, and physical chemists do not seem agreed that the Bjerrum unit is preferable.

*Equation XX.*  $pH$ .— $pH$  has been calculated by Hasselbalch's equation as given above (20). The derivation of the value 6.12 for  $pK'$  has been given under the discussion of Equation XIX.

#### DISCUSSION OF RESULTS.

Since the experimental data have already been referred to in the theoretical part of this paper, little discussion appears necessary at this point.

As stated in our theoretical discussion, since the application of the gas laws to electrolyte solutions is approximate rather than exact, deviations of a few per cent of their values between the  $\text{Cl}$  and  $\text{HCO}_3$  ratios, and of both from the calculated  $r$  values, would not be unexpected, nor incompatible with the soundness of the theoretical considerations. The agreement of the two ratios, and of both with the theoretical  $r$  values, indicated in Fig. 12, is as close as one might anticipate.

The  $r$  values calculated by Equations 10 and 14 respectively (at the bottom of section *d* of Tables VIII, IX, X, and XI) although theoretically equal, differ slightly in most cases, because they are based in part on different analyses, and subject, therefore, to different experimental errors. The value  $B_c$  occurs in Equation 14 but not in Equation 10.

The correspondence between the total values of the calculated and observed amounts of water in cells and serum is sufficient to demonstrate the validity of the assumption in Equation 4 on which the theoretical values are calculated, *viz.* that  $(\text{H}_2\text{O})_s : (\text{H}_2\text{O})_c = \Sigma (\text{M})_s : \Sigma (\text{M})_c$ .

<sup>9</sup> See Warburg (10), p. 259, Table XLIV.

The measurement of the water *shift* is, like the chloride ratio, one in which more data, by precise methods, are desirable. The water shifts between cells and serum observed as a result of the changes in CO<sub>2</sub> tension agree, however, except in Experiment 2, with the theoretically calculated shifts, as indicated in Fig. 13, about as closely as could be expected from the degree of accuracy of the methods and the relatively rather small shifts occurring within the pH range used. Taken together with the agreement between our calculated and Warburg's observed water shifts (observed by noting the change in cell oxygen capacities) reviewed in Table II, the shifts may be considered to be in fair accord with those predicted from the pH changes by Equations 23 and 24.

#### SUMMARY.

On the basis of the assumption that the laws holding in dilute solutions for (1) the relationships between the reaction and the amounts of base bound by weak and strong acids, (2) the distribution of diffusible and non-diffusible electrolytes on two sides of a membrane (Donnan), and (3) the proportionality between the ratio  $\frac{\text{molecules + ions of solute}}{\text{molecules of water}}$  and the osmotic pressure, are also valid for blood, mathematical expressions have been derived which predict the distribution of electrolytes and water between cells and serum, and the manner in which the distribution is affected by changes in pH and oxygen content. The effects of varying CO<sub>2</sub> tensions have been investigated, and the results have been found to approximate those predicted.

Calculations based on the data of Loeb, Atchley, and Palmer (22) indicate the probability that the same physicochemical laws defined for the cells-serum equilibria govern the distribution of electrolytes between blood serum and fluids in the serous cavities, although the permeabilities of the membranes are different and conditions are such that exact osmotic equality cannot apparently exist between the serum and fluids.

Applied to the methods for determining blood pH by the CO<sub>2</sub> capacity and dialysis methods, the data obtained have been used to estimate the corrections necessary because of the heterogeneous character of the blood in the former case, and the Donnan membrane effect in the latter.

In addition to supplying evidence concerning the validity of the theoretical considerations advanced in this paper, the experimental data demonstrate the following:

The base bound by the cell proteins of oxygenated horse blood over the physiological pH range is approximately expressed in milli-equivalents by the equation  $[BP]_c = 3.6 [Hb]_c$  ( $pH_c - 6.6$ ), when  $Hb$  expressed millimols of hemoglobin in terms of oxygen capacity. The  $[BP]_c$ ,  $pH_c$  curve is slightly concave towards the  $pH$  ordinate, so that the above linear equation is an approximate expression of the results.

The base bound by the serum proteins is indicated, over the physiological range of reaction, by the equation  $BP_s = 0.068 P_s$  ( $pH_s - 4.80$ ), where  $P_s$  expresses grams of serum protein.

The osmolar concentrations in blood cells and serum are equal when calculated as  $\frac{\text{molecules} + \text{ions of solutes}}{\text{water}}$ , the electrolytes being assumed to be equally dissociated in cells and serum.

Our thanks are due to Dr. A. B. Hastings and Dr. C. R. Harington for data on the base-binding power of the cell proteins, and to Dr. Hastings for the preparation of the nomogram of Fig. 6b.

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## THE PRESENCE OF AN YEAST GROWTH-PROMOTING VITAMINE IN CANE-SUGAR.\*

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Since the work of Wildiers (1), a number of investigators have confirmed the fact that certain extracts, which we now regard as being rich in water-soluble vitamines, will exert a marked stimulating action on the growth of yeast. Some of the later workers in the field, particularly Kurono (2), have associated this action with the presence of vitamine B in these extracts. Kurono found that an extract prepared from rice polishings and added to a Nägelei or Hayduck solution, accelerated the growth of various strains of yeast. With few exceptions, the yeast growth-promoting property of various vitamine extracts was generally regarded as being due to vitamine B until 1921, when Funk and Dubin (3) clearly demonstrated that the growth stimulation is due not to vitamine B, but to the presence of a newly discovered vitamine which is usually found associated with vitamine B in its natural source, and to which the term vitamine D was applied.

Whereas we have always been of the opinion that yeast is unable to grow without vitamine D, this view has not been shared by other investigators. Fulmer, Nelson, and Sherwood (4) described a medium composed of ammonium chloride, calcium chloride, dipotassium phosphate, calcium carbonate, dextrin, and cane-sugar (designated by them as medium F) in which they grew yeast for 2 years by making 300 successive transplantations without the addition of any other substances to the media. This work was confirmed by Fulmer and Nelson (5). Nelson, Fulmer, and Cessna (6) found that in an artificial diet for

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rats, ordinary brewer's yeast could be replaced by the yeast grown in medium F. Harden and Zilva (7) working with pigeons, also found that yeast grown on a synthetic diet, is able to synthesize vitamine B, but to a lesser degree than when grown in the presence of vitamine D. Eijkman, van Hoogenhuijze, and Derkx (8) found that yeast, grown in an artificial medium, and used as a source of vitamine B in feeding experiments with fowls, was devoid of this vitamine. In comparing their results with those of Nelson and Cessna, they drew the conclusion that vitamine B for birds and antineuritic vitamine for rats were two distinct substances.

MacDonald and McCollum (9) found that yeast does not require any specific substance for growth and Miss MacDonald (10) described the cultivation of yeast in a medium composed of 50 gm. of cane-sugar, 2 gm. of dihydrogen potassium phosphate, 2.35 gm. of ammonium sulfate, 0.25 gm. of calcium chloride, and 0.25 gm. of magnesium sulfate dissolved in a liter of water. Grown in the above medium, while the yields were not stated, they were large enough to be used for rat feeding experiments, and the yeast obtained proved to be an adequate source of vitamine B, although it had to be given in increased dosage. Heller (11), a collaborator of Fulmer and Nelson, found, however, that yeast grew in medium F only at incubator temperature ( $30^{\circ}\text{C}.$ ), that the yield was unsatisfactory and that the yeast contained less vitamine B as compared with yeast grown under normal conditions and used for rat feeding purposes.

From the above data it is not entirely clear whether or not yeast can synthesize vitamine B and whether or not yeast can grow in a medium devoid of vitamine D. Funk and Paton (12) were able to show that yeast, in growing, takes out the vitamine D from the medium with great avidity; and we have never observed a satisfactory growth in the absence of vitamine D. As we were desirous of preparing a large amount of yeast for feeding experiments with rats and pigeons, we at first used a simple synthetic medium such as that described by MacDonald. To our surprise, we obtained hardly any growth at all when we kept the inoculated flasks at room temperature as MacDonald recommended, and very little growth when the flasks were kept in an incubator at  $37^{\circ}\text{C}.$  It soon became evident to us that we would

never be able to obtain enough yeast by this method to carry on feeding tests for any length of time.

A significant fact, however, which we observed at this time, led us to investigate the problem of yeast growth more closely. We observed in the course of some sixteen transplantations of yeast in a MacDonald medium, that the yield of yeast was practically constant and did not decrease as would be expected from the gradual dilution of the vitamine D present in the initial inoculation. We therefore came to the conclusion that the medium used must contain a yeast growth-promoting substance which was present in a constant amount in each fresh medium to which the transplantations were made. As the medium is composed of four inorganic salts, cane-sugar, and distilled water, it was an easy matter to find the guilty ingredient or ingredients. As we expected, it proved to be the cane-sugar. This was not at all surprising to us as we had previously found and described in a recent publication (13) that other supposedly pure food substances such as gelatin and casein contained a vitamine which promoted the growth of yeast and certain bacteria.

After eliminating the salts and distilled water as possible factors, we found that the growth of yeast was negligible when grown in a synthetic medium in which the cane-sugar used was purified by shaking a solution of it with fullers' earth and recovering the sugar, or by recrystallization from alcohol, when the inoculation was made from a starved culture grown in a medium containing non-purified sugar. The growth of yeast, therefore, depends on two factors; on the vitamine D found in the medium, and on the amount of vitamine D introduced with the yeast cells used for inoculation.

The following experiments prove beyond a doubt that the strain of yeast, which we at least have used, is not only unable to synthesize vitamine B on a purified medium, but is also unable to grow in total absence of vitamine D. As soon as vitamine D is added to the medium, the synthesis of vitamine B proceeds in a normal way. The question arises as to how far our findings will apply to other strains of yeast. We cannot at present answer this question with any certainty, but from the work of Kurono (2) who investigated eighteen different strains of yeast and found among them only four that were not influenced in their growth

by the addition of vitamine, we can conclude that we are perhaps dealing with a principle which is generally adaptable. The four strains of yeast, which were found to be exceptions to the rule, should be reinvestigated from this new standpoint. As Kurono also dealt with ordinary cane-sugar, the various strains of yeast either showed smaller requirements of vitamine D or else exceedingly large amounts of this substance were introduced with the first inoculation. Our findings emphasize once more the evident importance of the "infinitely little" in nutritional experiments; and that commercially pure substances, like cane-sugar, should not be used in experiments in nutrition without further purification.

#### EXPERIMENTAL.<sup>1</sup>

To illustrate the exceedingly small yields obtained on the medium described by MacDonald, the following experiment was performed. 1,800 cc. of this medium, which we will call ordinary medium, and designate as "medium O", were divided into 12 Erlenmeyer flasks holding 150 cc. each, and each flask was seeded with 5.0 cc. of a yeast suspension from a 14th subculture. After incubating at 37°C. for a month, the yield of yeast amounted to 1.5 gm. dry; whereas on addition of 1 cc. of autolyzed yeast to each of six flasks containing 150 cc. each and similarly seeded, we obtained after 4 days incubation, 3 gm. of dried yeast. When the cultures were examined under the microscope, a significant difference was observed. Whereas in the first case the cells were brownish red in color and presented a shrunken spore-like appearance, in the second case they were almost colorless and in active budding.

The subculturing of the yeast was carried out by introducing, with a sterile pipette, 5 cc. of yeast suspension in medium O into 150 cc. of fresh medium. All the inoculations described in this paper were done with the 14th and 15th subcultures. As we have stated in the introduction, the yields obtained in each successive inoculation were so comparable that we suspected a vitamine-like impurity in one or more of the ingredients. In order to definitely determine the source of this vitamine, we purified all the ingredients which made up the medium. Each salt was

<sup>1</sup> This work was started in conjunction with Miss Olive Sheets.

dissolved separately in water and shaken up with a definite quantity of fullers' earth, the same procedure being applied to cane-sugar (Domino brand). After filtration the solutions were evaporated to dryness and the salts bottled and kept free from moisture and dust. The sugar residue was recrystallized once from alcohol previous to use. The distilled water was redistilled three times in an all glass apparatus. All the tests were made in uniform test-tubes, 10 cc. of the nutritive solution being inoculated with 0.5 cc. of yeast suspension from the 14th subculture. After a suitable period of incubation the yeast was killed by heat and the yeast growth measured in millimeters by the centrifuge method used in our laboratory. In the first experiment, where

TABLE I.  
*Effect of Purified Media on the Growth of Yeast.*

No.	Medium.	Growth after	
		3 days. mm.	4 days. mm.
1	Medium consisting of all purified ingredients (medium P).....	1.5	1.5
2	Medium P with ordinary sugar replacing purified.....	5.0	5.0
3	" " " salts " " .....	1.5	1.5
4	" " " water " " .....	1.5	1.5
5	" O + 1 drop autolyzed yeast. Viability test....	25.5	23.5
6	10 cc. sterile water + 0.5 cc. yeast suspension.....	1.4	1.5
7	Same as No. 6, but with yeast killed before incubation.....	1.0	1.0

yeast was grown at 37°C. for 3 and 4 days, respectively, the effect of purification of all the ingredients used, was studied. The results of this experiment are embodied in Table I.

These results prove that the cane-sugar was the only vitamin D-bearing factor in the medium and hence to this factor our whole attention was directed. The cane-sugar was recrystallized three times from alcohol and a fraction from each successive crystallization was tested in conjunction with the usual salts and distilled water used in medium O. The same yeast suspension was used as in the previous experiment and the tests were carried out in the same way. The tubes were incubated for 3 days at 37°C. In this experiment we also studied the effect

of the addition to purified medium of the ash obtained from 1 gm. of ordinary sugar.

The experiment tabulated in Table II shows that the vitamine-like impurity in cane-sugar can be practically eliminated after one crystallization from alcohol, and entirely after shaking with fullers' earth and then recrystallizing from alcohol; and that the yeast growth-promoting substance in cane-sugar is not likely to be of an inorganic nature. The sugar obtained after three recrystallizations from alcohol was polarized and the polarimetric reading found to be almost identical with that of ordinary sugar before recrystallization from alcohol, showing that the sugar was not altered. (The rotations were  $[\alpha]_D^{20} = +66.75$  and

TABLE II.  
*Effect of Recrystallization of Cane-Sugar.*

No.	Medium.	Growth after 3 days. mm.
1	Medium O.....	6.12
2	" " with once recrystallized sugar replacing ordinary sugar.....	2.5
3	Medium O with twice recrystallized sugar replacing ordinary sugar.....	2.37
4	Medium O with thrice recrystallized sugar replacing ordinary sugar.....	2.12
5	Medium O + 1 drop autolyzed yeast.....	23.0
6	" P + ash from 1 gm. of ordinary sugar.....	2.12
7	10 cc. sterile water + 0.5 cc. yeast suspension (killed)....	2.0

+66.91, respectively, in 11.78 per cent solution.) This polarization experiment also shows that the removal of the vitamine-like impurity has no effect on the rotation of the sugar.

We also carried out an experiment in which the weights of yeast, grown in an ordinary and a purified medium, were determined. Four flasks, each containing 100 cc. of medium O, and four flasks each containing 100 cc. of medium P, were inoculated with 5.0 cc. of yeast suspension from a 15th subculture in medium O. The flasks were kept in an incubator at 37°C. for 2 weeks, two flasks of each series were combined and then filtered on weighed paper filters and after thoroughly washing out the salts, the filter papers were dried to constant weight. The

weights in milligrams of yeast cells were as follows: On medium P, the dried yeast weighed 23.0 and 26.2 mg., respectively, giving an average of 24.6; while in medium O, the growth of yeast amounted to 68.4 and 70.6 mg., respectively, with an average weight of 69.5 mg. This confirms the growth obtained in Table I and measured by the centrifuge method, and shows that in medium P, the growth is approximately one-third of that in medium O.

As the yeast growth-promoting substance could be almost entirely removed from cane-sugar by one recrystallization from alcohol, it was of interest to see if this substance could not be removed from the alcohol mother liquors and its effect on yeast growth determined. We therefore evaporated the alcoholic

TABLE III.  
*Effect of Impurities Obtained from Cane-Sugar on Yeast Growth.*

No.	Medium.	Growth after 66 hrs. mm.
1	Medium O.....	4.25
2	" P.....	1.5
3	" "+ 0.25 cc. solution R.....	3.5
4	" "+ 0.5 " " "	3.65
5	" "+ 1.0 " " "	3.0
6	Control. 10 cc. sterile water + 0.5 cc. yeast suspension..	1.25
7	" Same as No. 6, but with yeast cells killed before incubation.....	1.0

mother liquors obtained from the first crystallization of the sugar, to a small volume *in vacuo*. The sugar which crystallized out was filtered off and the filtrate evaporated to a syrup. This syrupy residue was then extracted with cold alcohol, the alcoholic solution filtered, and the filtrate evaporated *in vacuo* to dryness. This residue was then dissolved in 20 cc. of water and sterilized, and the solution designated as "solution R." This solution was found to be active for the growth of yeast when added to 10 cc. of medium containing purified sugar (medium P) in amounts up to 0.5 cc., but the activity diminished when 1 cc. and larger amounts were added, indicating the presence of inhibiting impurities in the solution.

These results are detailed in Table III.

As we have stated before, one important factor in the growth of yeast in a synthetic medium is the source of the yeast used for inoculation. When the yeast cells used for seeding are taken from a medium poor in vitamine D as medium O, and transferred to a purified synthetic medium, the growth of yeast is almost nil, whereas if the seeding cells are taken from a vitamine D-rich medium, such as an agar-malt culture, the growth of yeast is appreciable. In the following experiment, the results of which are detailed in Table IV, the inoculating yeast cells were obtained

TABLE IV.

*Effect, on Yeast Growth, of Inoculation with Yeast Cells from a Vitamine D-Poor and Rich Culture.*

No.	Medium.	Growth after 66 hrs.	
		Total. mm.	Net. mm.
1	Medium P (inoculated with 1 cc. Nägelei agar-malt suspension).....	0.5	
2*	Medium P (inoculated with 2 cc. Nägelei agar-malt suspension).....	2.25	2.25
3	Control. Sterile H <sub>2</sub> O inoculated with 1 cc. Nägelei agar-malt suspension and killed before incubation.....	0.0	
4	Control. Sterile H <sub>2</sub> O inoculated with 2 cc. Nägelei agar-malt suspension and killed.....	0.0	
5*	Medium P (inoculated with 0.5 cc. from 14th subculture).....	2.25	0.25
6	Control. Sterile H <sub>2</sub> O inoculated with 0.5 cc. from 14th subculture and killed before incubation.....	2.0	

\* In Nos. 2 and 5, the respective blank controls were subtracted to give the net growth.

from a vitamine D-starved culture (a 14th subculture in medium O), the same as that used in the previous experiments; and the other was taken from a suspension prepared by shaking 100 cc. of a Nägelei solution with a loopful of fresh yeast from an agar-malt culture.

From Table IV, it can be seen that when the inoculation is made from a starved culture the net growth of yeast cells is only 0.25 mm., while when the inoculation used was taken from a vitamine D-rich culture, the growth is almost tenfold or 2.25 mm.

Ordinary cane-sugar contains a vitamine-like substance which promotes the growth of yeast cells, and which is probably identical with vitamine D. We believe that other commercially prepared substances used in bacterial cultures and animal feeding experiments, such as starch, dextrin, glucose, etc., contain similar vitamine-like substances as impurities.

It is, therefore, essential when using cane-sugar in a synthetic medium, to purify the sugar by shaking its solution with fullers' earth and recrystallizing from alcohol or by direct crystallization from alcohol. Although yeast cannot grow in an absolutely pure synthetic medium devoid of vitamine D, the amount of this last substance carried over by the inoculation is often enough to promote growth and hence give misleading results.

The source of the inoculating cells should, therefore, be taken into account. Yeast grown in a synthetic medium, poor in vitamine D, cannot be used as a good source of vitamine B for animal feeding experiments, as the yeast cannot synthesize vitamine B in the absence of vitamine D.

#### CONCLUSIONS.

1. The growth of yeast is dependent on two factors: The vitamine-like substance present in one or more of the ingredients making up the medium, such as cane-sugar; and the amount of vitamine carried over by the inoculation.
2. The growth of yeast is dependent on the source of the inoculating yeast cells, whether it is obtained from a vitamine-rich or a vitamine-poor culture.
3. Yeast cannot synthesize vitamine B in the absence of vitamine D for the reason that in the absence of the latter, yeast cannot grow.
4. Our conclusions emphasize the great importance that minute amounts of undetected vitamines may have on experiments in animal nutrition.

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## THE AMINO-ACID NITROGEN OF THE BLOOD.

### I. THE TOTAL FREE AMINO-ACID NITROGEN IN BLOOD.

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The rapid development of simple and convenient methods of blood chemistry and their ready application to routine procedure on a large scale has resulted in the accumulation, within the last decade, of a huge mass of data concerning the concentration of all the known non-protein nitrogenous constituents of the human blood except that of the amino-acids. That progress along this line was less rapid is due to the fact that the method heretofore available for the estimation of amino-acid nitrogen is somewhat complicated and time-consuming. It does not therefore fit into the program of the clinical or hospital laboratory. Moreover, the relatively few determinations of the amino-acid nitrogen that have been made on pathological bloods have not yielded results of any great clinical significance. For these reasons the quantitative study of the amino-acid nitrogen of the human blood still belongs to the domain of special investigation. The work to be reported was started about 2 years before the recent publication of Folin's (1) colorimetric method for the estimation of the amino-acid nitrogen in the blood.

Since the work of Bock (2) has established the amino-acid nitrogen value of normal human blood, it was planned to investigate the amino-acid nitrogen in the blood from hospital patients. Observations have also been made on the basic amino-acids and on the conjugated amino-acids or peptide nitrogen of the protein-free blood filtrates. These studies will be dealt with in subsequent papers.

The bloods were prepared for analysis by the gasometric method of Van Slyke essentially as recommended by Bock (2). The bulk of the proteins was removed by heat-acetic acid coagulation, and the last traces of protein were removed with trichloroacetic acid in a concentration of 4 to 5 per cent.

Studies on the relative merits of various methods for the precipitation of blood proteins were reported recently by Sjollema and Hetterschy (3), Richter-Quittner (4), Wolff (5), Fischer (6), and Hiller and Van Slyke (7). The claim of Bock that boiling with dilute acetic acid does not cause a measurable hydrolysis of the blood proteins was confirmed by Brun (8), Fischer (6), Mukai (9), Okada (10), and Richter-Quittner (4). That trichloroacetic acid in proper concentration is a suitable precipitant for blood proteins was shown by Greenwald, Bock, and more recently, by Hiller and Van Slyke. In the Bock procedure for the estimation of the amino-acid nitrogen of the blood, the trichloroacetic acid is removed from the filtrate by boiling in an open vessel. Hiller and Van Slyke observed that a 5 per cent trichloroacetic acid filtrate of a solution of Witte's peptone, when boiled, yielded an increased amount of amino-acid nitrogen due to the hydrolysis of the protein cleavage products by the acid. This source of error could be eliminated by diluting the filtrate with an equal amount of water. A 2.5 per cent solution of trichloroacetic acid does not apparently possess such power of hydrolysis. In our experience with blood filtrates, we have not been able to find any difference in the amino-acid nitrogen value of a 5 per cent trichloroacetic acid filtrate whether boiled with or without previous dilution with water. For greater accuracy, we have not used any urease for the destruction of urea, but have corrected the initial gas volumes for the nitrogen from the slowly reacting amines by the somewhat lengthy time-factor method of Levene and Van Slyke. This procedure was necessitated by the observation that urease preparations contain appreciable amounts of free amino-acids and protein cleavage products which escape precipitation with trichloroacetic acid and that treatment of the blood with urease does not entirely eliminate the error due to non-amino compounds. As a matter of fact, some blood filtrate in which the urea was destroyed by hydrolysis with acid yielded the same volume of slowly evolving nitrogen as an untreated portion of the same fil-

trate. The possible significance of this finding, interesting in itself, would bear closer investigation. We have therefore collected at random from our large mass of data a few illustrative figures (Table I). The reaction time was uniformly 4 minutes.

TABLE I.

*Volume of Gas from Slowly Reacting Amines in Blood Filtrates after Decomposition of Amino-Acids with Nitrous Acid.*

Species.	Diagnosis.	Volume of blood an- alyzed.	Gas volume of un- treated blood filtrate.			Gas volume of hydro- lyzed blood filtrate.	Gas volume of urease treated blood.
			cc.	cc.	cc.		
Beef.	.	4.85	0.12	0.11	0.14		
		5.00	0.16	0.16	0.25		
		5.00	0.12	0.14	0.16		
		5.00	0.18	0.10	0.16		
		5.00	0.10	0.10	0.13		
		5.00	0.14	0.09	0.19		
Dog.	Peptone shock.....	4.82	0.14	0.20			
Human.	Uremia.....	3.24	0.19	0.21	0.20		
	Normal.....	4.00	0.12	0.12			
	" .....	3.64	0.07	0.08			
	Gas poisoning.....	3.00	0.15	0.15	0.18		
	Hypertension.....	3.00	0.11	0.10			
	Chronic interstitial nephritis.....	3.50	0.19	0.15			
	" " " .....	2.10	0.87	0.33			
	Hypertension.....	4.50	0.15	0.06			
	" .....	3.00	0.11	0.11			
	Asthma.....	3.64	0.11	0.14			
		4.00	0.18	0.06			
	Aortic regurgitation.....	3.20		0.20			
	Hypertension.....	3.50		0.26			

As may be seen from the table, the blank figures in urea, ammonia, and peptide nitrogen-free filtrates are quite large and a failure to apply a correction for them may lead to serious error. In a few instances we found that urease or acid hydrolysis were equally ineffective in removing all of the substances that react slowly with nitrous acid. No positive statement as to the exact nature of these bodies is warranted at the present time. The

TABLE II.  
*Amino-Acid Nitrogen in Blood.*

All figures represent milligrams of nitrogen per 100 cc. of blood.

Diagnosis.	Total non-protein nitrogen.	Amino-acid nitrogen.
Normal.....		4.28
" .....		5.30
" .....		4.70
Alcoholic coma.....		5.85
" acute.....		4.55
" neuritis.....		4.85
Anginoid pains of heart.....		6.29
Aortic regurgitation.....		6.25
Asthma, bronchial.....		6.07
" " .....		3.10
" " .....		5.39
Arthritis.....		5.38
" .....		6.44
Carbon monoxide poisoning.....		5.68
" " " .....		5.37
" " " .....		4.33
Hemiplegia, hypertension.....		5.68
" .....		7.08
" .....		4.39
" hypertension.....		4.62
Hypertension, chronic interstitial nephritis.....		5.73
" lues, hemiplegia .....		4.62
" acute alcoholism.....		7.21
" " " .....		5.24
" " " .....		5.34
" " " .....	170.80	9.67
" " " .....	170.50	7.66
" " " .....	64.00	5.60
" .....		3.86
Nephritis, interstitial.....		5.20
Diabetes.....		4.89
" .....		5.14
" .....		3.31
Epilepsy.....		5.89
" .....		3.83
Pneumonia.....		5.03
" .....		3.82
Pernicious anemia.....	23.00	3.64
Uremia.....		4.12
Unknown.....		5.62
" .....	44.70	4.72

TABLE II—*Concluded.*

Diagnosis.	Total non-protein nitrogen.	Amino-acid nitrogen.
Beef blood.....		4.60
		4.52
		3.80
		5.29
		5.01
		5.54
		3.99
		3.76
		5.38
		5.54
		5.23
		4.97
		5.02
		4.74
Sheep blood.....		4.60
		4.93
Dog blood. (Peptone shock.).....		3.54
(Ether anesthesia.).....		5.32
( " " ).....		4.87
( " " ).....		4.10

basic character of at least some of them seems to be indicated by the considerable reduction in the size of the blank, as a result of phosphotungstic acid treatment of the filtrate. We hope to be able to enter into this problem more thoroughly in the future.

Table II shows the figure for amino-acid nitrogen of the blood obtained by the method outlined above. Analyses were usually made in triplicate. The values are somewhat lower than those reported by Bock and others (Snoo (11) and Okada (10)) who followed his technique. The figures for beef blood agree with those obtained by Cary (12), while those for human blood come close to the values reported by Hammett (13) on the tungstic acid filtrates of 60 bloods. Most of our figures are around 5.00 mg. The highest figures, 7.00 to 9.00, were obtained in cases of nephritis and conditions associated with circulatory disturbances.

## SUMMARY.

Attention has been drawn to certain sources of error in the determination of the amino-acid nitrogen of the blood by the Bock-Van Slyke method. Amino-acid nitrogen values in a number of bloods are reported.

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## THE AMINO-ACID NITROGEN OF THE BLOOD.

### II. THE DIAMINO NITROGEN IN THE PROTEIN-FREE BLOOD FILTRATE.

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The literature affords no definite data on the quantity of nitrogen occurring in the blood of any species in the form of diamino-acids. Brun (1) has expressed some doubt as to the occurrence of hexone bases in human blood. Abel (2) found histidine in the dialysate from the circulating blood of the dog. Abderhalden (3) reports the identification of arginine, histidine, and lysine in the dialysate from beef blood.

In the present investigation we have determined the free basic amino nitrogen in a number of samples of human blood. The method employed is an adaptation of the Hausmann procedure, in which the basic amino-acids are precipitated with phosphotungstic acid. The figure for basic amino-acid nitrogen is obtained by difference, the amino-acid nitrogen being determined in the blood filtrate before and after treatment with phosphotungstic acid in hydrochloric acid.

#### EXPERIMENTAL.

The adequacy of the procedure for the quantity of basic amino-acids which might be expected to occur in blood was tested by application to dilute solutions of mixed amino-acids obtained from the hydrolysis of casein. Casein was hydrolyzed with 20 per cent hydrochloric acid. The total nitrogen of the solution was determined. The ammonia and humin were removed with the aid of lime in the usual manner and the nitrogen of the filtrate

was estimated before and after treatment with a 10 per cent solution of phosphotungstic acid in 2 per cent hydrochloric acid. The nitrogen estimations were made by a micro Kjeldahl method. The solution was digested with 2 cc. sulfuric acid, the ammonia distilled through a Liebig condenser into 0.01 N acid, and the latter titrated with dilute alkali. The results are summarized in Table I.

By comparing our figures for the percentage of diamino nitrogen in casein with some of those found in the literature, it may be seen that the Hausmann method is capable of yielding results of a high degree of accuracy even with dilute solutions of basic amino-acids. Osborne and Harris (4), using their modification of the

TABLE I.

*Percentage Determination of Diamino Nitrogen in Dilute Solutions of Casein.*

Figures for nitrogen represent milligrams per 100 cc. of solution.

Volume of solution taken.  cc.	Total nitrogen.  mg.	After removal of ammonia and humin nitrogen.		Diamino nitrogen.  mg.	Diamino nitrogen of total nitrogen.  per cent
		Nitrogen before treat- ment with phospho- tungstic acid.  mg.	Nitrogen after treat- ment with phospho- tungstic acid.  mg.		
10	12.16	10.92	8.12	2.80	23.02
20	1.99	1.762	1.31	0.452	22.71
5	25.61	22.96	17.22	5.74	22.41
5	63.68	57.12	42.00	15.12	23.90

Hausmann procedure, found 23.75 and 22.35 per cent diamino nitrogen. Using his own nitrogen distribution method, Van Slyke (5) found 23.92 per cent of nitrogen in the form of histidine, arginine, and lysine, while the method of Kossel and Kutcher gave 23.00 per cent of nitrogen from the hexone bases of casein. Our figures average 23.01 per cent nitrogen from these bodies.

The procedure for the determination of the free diamino nitrogen of the blood was carried out as follows:

25 to 40 cc. of blood were precipitated by the heat-trichloroacetic acid method of Bock (6). The filtrate was slowly boiled for the removal of the trichloroacetic acid. The solution was then acidified with 2 cc. of concentrated hydrochloric acid and diluted

TABLE II.  
*Occurrence of Diamino Nitrogen in Blood.*

All figures represent milligrams of nitrogen per 100 cc. of blood.

Diagnosis.	Amino-acid nitrogen before precipitation with phospho- tungstic acid.	Amino-acid nitrogen after precipitation with phospho- tungstic acid.	Diamino nitrogen.
	mg.	mg.	mg.
Normal.....	4.72	2.81	1.91
" .....	4.35	2.62	1.73
Alcoholic coma.....	5.85	3.04	2.81
" "	8.59	4.39	4.20
" acute.....	4.55	2.82	1.73
" neuritis.....	4.85	3.88	0.97
Anginoid pains of heart.....	6.94	5.47	1.47
Not given.....	5.62	4.36	1.26
Arthritis.....	5.38	4.13	1.25
Asthma, bronchial.....	6.07	3.95	2.12
Arthritis.....	6.44	4.16	2.28
Asthma, bronchial.....	3.10	3.30	
" "	5.39	4.01	1.38
Aortic regurgitation.....	6.25	5.53	0.72
Anemia, pernicious.....	3.64	3.51	0.13
Cardioneuritis.....	5.20	3.15	2.05
Diabetes.....	5.14	2.82	2.32
" .....	3.31	3.24	
" .....	4.89	3.46	1.43
Epilepsy.....	5.89	2.69	3.20
Carbon monoxide poisoning.....	5.68	3.76	1.93
" " "	4.33	3.21	1.12
" " "	5.37	3.72	1.65
Hemiplegia.....	7.08	4.90	2.18
" .....	4.39	2.14	2.25
Hypertension.....	7.12	2.58	4.54
" chronic interstitial nephritis.....	5.73	2.37	2.36
Hypertension.....	5.68	3.73	1.95
Lues, hemiplegia, hypertension.....	4.62	4.63	
Hypertension, bronchopneumonia.....	3.82	2.90	0.92
" .....	5.34	4.10	1.24
" .....	7.66	4.13	3.53
Acute alcoholism.....	5.60	4.03	1.57
Hypertension.....	3.86	2.98	0.88

TABLE II—Concluded.

Diagnosis.	Amino-acid nitrogen before precipitation with phospho- tungstic acid.	Amino-acid nitrogen after precipitation with phospho- tungstic acid.	Diamino nitrogen.
	mg.	mg.	mg.
Pneumonia.....	5.03	3.03	2.00
Uremia.....	4.12	3.32	0.80
Beef blood.....	5.54 2.99 5.75	5.07 2.43 4.81	0.47 0.56 0.94
Dog blood.....	5.33 3.54	4.01 1.64	1.32 1.90

to 40 cc. in a graduated cylinder. 20 cc. were neutralized and boiled down to a small volume for the estimation of the total free amino-acid nitrogen. 20 cc. were treated with 5 cc. of the phosphotungstic acid reagent and allowed to stand for 48 hours at room temperature. The precipitate was separated by filtration and an aliquot of the filtrate, usually 20 cc., was made slightly alkaline to phenolphthalein, then just acidified with acetic acid and concentrated to a small volume for analysis in the Van Slyke apparatus. The difference between the two values, properly corrected for blanks, gave the quantity of diamino nitrogen in the blood filtrate.

#### DISCUSSION.

Table II shows the results obtained by the method when applied to blood. Diamino nitrogen was found in all but three of the bloods analyzed. The quantity varied from 0.13 to 4.54 mg. per 100 cc. of blood. The lowest figure probably falls within the limits of experimental error. Most of the values are larger than 1.0 mg., constituting from about 20 to 60 per cent of the total free amino-acid nitrogen. One blood from a case of alcoholic coma shows an amino-acid nitrogen of 8.59 mg., about 50 per cent of which is in the form of diamino nitrogen. But a high amino-acid nitrogen is not always accompanied by an increased diamino nitrogen. There seems to be no constant relationship between the two values as far as can be seen from the table.

## SUMMARY.

It has been shown that the Hausmann method is applicable to the precipitation of small amounts of the hexone bases from dilute solutions of amino-acid mixtures. A method for the estimation of the free diamino nitrogen of the blood has been described. Values for diamino nitrogen, ranging from 0.13 to 4.54 mg. per 100 cc., are given in a number of bloods.

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## THE AMINO-ACID NITROGEN OF THE BLOOD.

### III. A STUDY OF THE OCCURRENCE OF PEPTIDE NITROGEN IN THE BLOOD.

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It is well known that the sum of the nitrogen from the various constituents of a protein-free filtrate falls short, to a variable extent, of the total nitrogen. In the bloods of 50 normal children, Leopold and Bernhard (1) found from 8.6 to 23.3 mg. of residual nitrogen per 100 cc. of blood. These figures included also the nitrogen from the amino-acids. In a number of instances the sum of the nitrogen from the known blood constituents amounted to no more than 50 per cent of the total nitrogen. Foster (2) claimed that in nephritic blood the sum of the nitrogen from the various bodies may fall to less than 50 per cent of the total nitrogen.

Woods (3) found considerable amounts of residual nitrogen in the alcoholic filtrates from the bloods of eighteen cases of nephritis, from 1.52 to 31.80 mg. per 100 cc., most of the values being around 8 to 10 mg. The total non-protein nitrogen varied from 26.77 to 138.10 mg. The latter figure was also accompanied by the highest amino-acid nitrogen (16.32 mg.) and the greatest amount of undetermined nitrogen (31.56 mg.) of the series. The height of the figures for residual nitrogen seemed in some cases to parallel the severity of the disease.

Large increases of total nitrogen and of residual nitrogen in the plasma and cells of the bloods from wound-shocked soldiers were reported by Duval and Grigaut (4). The urea nitrogen in these cases remained practically normal, while the residual nitrogen rose

in one case, as high as 67.30 mg. Cases with fatal termination exhibited, during the course of the disease, a persistently high residual nitrogen until death.

No great effort has hitherto been made to ascertain the nature of the substances responsible for this undetermined nitrogen. Schweriner (5) failed to note an increase in the formaldehyde index of the hydrolyzed filtrates from normal bloods, obtained by the precipitation of the proteins with mercuric chloride in an acid solution. He found small amounts (3 to 4 mg.) of peptide nitrogen in the bloods from a limited number of cases of nephritis, and from 6.0 to 8.0 mg. per 100 cc. in cases of cancer. Wolff (6) estimated the "albumoses" in the blood by the difference in the nitrogen of the filtrate from trichloroacetic acid and phosphomolybdic acid precipitation. Cases of croupous pneumonia showed more than 9.0 mg. of albumose nitrogen. Slight amounts were found in pulmonary tuberculosis, typhoid fever, and cases of lung abscess.

It should be pointed out that the methods used by the two last named investigators are not free from objection. The mercuric chloride used by Schweriner for the precipitation of the proteins might have also precipitated polypeptides. The low figures for total non-protein nitrogen, 22 to 27 mg., obtained by this author on the mercury-free filtrates seem to justify this suspicion. In the procedure of Wolff, the phosphomolybdic acid employed, besides precipitating albumoses, may also remove basic amino-acids. A reading of this author's paper does not give one the impression that a careful study has been made of the sharp selective precipitation of these so called albumoses by phosphomolybdic acid.

Wu (7) found but insignificant amounts of undetermined nitrogen in the tungstic acid filtrates from plasma, while the corpuscles contained an average of 20.0 mg. per 100 cc. This is ascribed to the probable presence of peptides and peptones in the blood cells. Folin and Berglund (8) have tabulated their data on the nitrogen partition in the bloods of twelve normal young men bled after a night's fast. The average undetermined nitrogen was 6.7 mg. in the plasma, 24.7 mg. in the corpuscles, and 13.7 mg. in the whole blood. These investigators venture the suggestion that the unknown nitrogenous material is partly made up of histones.

In order to find out whether protein intermediate products could account to any extent for the undetermined nitrogen, we have estimated the quantity of peptide nitrogen in the filtrates from a number of bloods.

#### EXPERIMENTAL.

40 to 80 cc. of blood were coagulated with heat and acetic acid, and enough trichloroacetic acid was added to make a 5 per cent solution of the reagent (Bock). After the removal of the trichloroacetic acid, the solution was made up to 80 cc. in a graduate containing 4 cc. of concentrated hydrochloric acid. 20 cc. were used for the determination of the total free amino-acid nitrogen; 20 cc. were treated with phosphotungstic acid for the determination of the free diamino nitrogen; 40 cc. were mixed with an equal volume of concentrated hydrochloric acid and boiled under a reflux condenser for 24 hours. The acid and then the ammonia were distilled off under reduced pressure; the neutral solution was then washed into a graduate containing 2 cc. of concentrated hydrochloric acid and made up to 40 cc. 20 cc. were treated with phosphotungstic acid for the determination of the peptide diamino nitrogen; the remainder of the solution was concentrated and analyzed for total amino-acid nitrogen.

Hiller and Van Slyke (9) claim that trichloroacetic acid in concentrations greater than 2.5 per cent precipitates some peptide nitrogen from beef blood. They, therefore, recommend the use of 2.5 per cent trichloroacetic acid with solutions containing proteins and their intermediate products. Greenwald (10) found that 2.5 per cent of trichloroacetic acid did not remove the last traces of protein from blood. He also stated that, according to some investigators, beef blood is not completely deproteinized only by a 10 per cent solution of the reagent. We have not been able to obtain water-clear and absolutely protein-free filtrates of fresh defibrinated beef blood with 2.5 per cent trichloroacetic acid.

#### DISCUSSION.

Table I gives the data from which the total peptide nitrogen and the diamino fraction thereof were calculated. The figures for total peptide nitrogen (Column G) are calculated by difference

TABLE I.  
*Peptide Nitrogen in Blood.*

All figures represent milligrams of nitrogen per 100 cc. of blood.

No.	Diagnosis.	Before hydrolysis.			After hydrolysis.			
		Total non-protein nitrogen. (A)	Total free amino-acid nitrogen. (B)	Free diamino nitrogen. (C)	Total amino-acid nitrogen. (D)	After treatment with phosphotungstic acid. (E)	Total diamino nitrogen. (F)	Total peptide nitrogen. (G)
	Normal.	4.27	1.46	5.16	3.47	1.69	0.89	0.23
	"	4.28		5.14	4.12	0.98	0.86	
	"	5.3	1.3	6.17	4.26	1.89	0.87	0.59
1	Alcoholic coma.	5.85	2.81	10.53	7.44	3.09	4.68	0.28
2	" "	30.0*	8.59	4.20	13.05	8.60	4.45	4.46
3	" acute.	4.55	1.73	9.77	6.79	2.98	5.22	1.25
4	" neuritis.	4.85	0.97	8.88			4.03	
5	Anginoid pains of heart.	6.94	1.47	9.37			2.43	
6	Aortic regurgitation.	48.0*	6.25	0.72	8.74	7.28	1.46	2.51
7	Asthma, bronchial.	3.10	0.00	7.6	5.51	2.09	4.5	2.09
8	" "	5.39	1.38	5.94			0.55	
9	" "	6.07	2.12	11.60	6.86	4.73	5.55	2.61
10	Arthritis.	5.38	1.25	7.16			1.78	
11	"	6.44	2.28	7.74	4.98	2.62	1.3	0.34
12	Diabetes.	34.5*	4.89	1.43	7.15	3.65	3.5	2.26
13	"	5.14	2.32	7.18	3.44	3.74	2.04	1.41
14	"	31.00*	3.31	0.07	3.41	3.34	0.1	0.04
15	Epilepsy.	48.0*	3.33		8.14	6.90	1.24	4.81
16	"	5.89	3.20	6.47			0.58	
17	Carbon monoxide poisoning.	5.68	1.93	10.34	8.93		4.65	
18	Carbon monoxide poisoning.	5.37	1.65	10.36	6.89	3.47	4.99	1.82
19	Carbon monoxide poisoning.	4.33	1.12	7.44	4.39	3.05	3.11	1.93
20	Hypertension.	5.68	1.95	9.78	7.28	2.50	4.1	0.55
21	Hemiplegia.	7.08	2.18	9.25	6.83	2.43	2.17	0.25
22	"	4.39	2.25	7.80	5.15	2.65	3.41	0.40

\* The figures represent non-protein nitrogen determinations made on tungstic acid filtrates. All other non-protein nitrogen figures were obtained on trichloroacetic acid filtrates.

TABLE I—Concluded.

No.	Diagnosis.	Before hydrolysis.			After hydrolysis.			
		Total non-protein nitrogen. (A)	Total free amino-acid nitrogen. (B)	Free diamino nitrogen. (C)	Total amino-acid nitrogen. (D)	After treatment with phosphotungstic acid. (E)	Total diamino nitrogen. (F)	Total peptide nitrogen. (G)
23	Hypertension.	42.0*	5.73	2.36	13.64	9.32	4.32	7.91
24	"		4.62	0.00	8.91	8.77	0.14	4.29
25	"		7.12	4.54	9.12			2.0
26	"	46.0*	5.24		8.5			3.23
27	" Bronchopneumonia.	23.5*	3.82	0.92	6.83	3.92	2.91	3.01
28	Hypertension.	28.5*	5.34	1.24	9.54	6.44	3.1	4.2
29	"	170.8	9.67		10.59			0.92
30	"	64.0	5.6	1.57	11.18	7.60	3.58	5.58
31	"	107.5	7.66	3.53	12.35	6.63	5.72	4.69
32	"	74.9	3.86	0.88	8.78	5.59	3.19	4.92
33	Cardionephritis.			5.2	2.05	9.89	5.76	4.13
34	Pernicious anemia.	23.00	3.64	0.13	4.82	4.46	0.36	1.18
35	Senility. Bronchopneumonia.			5.03	2.00	6.02	3.04	2.99
36	Uremia.	50.0	4.12	0.80	7.52	5.72	1.74	3.40
37	Unknown.			5.62	1.25	6.31	4.90	1.41
	Beef blood.			5.00		6.97		1.97
				5.54		0.49		1.09
				4.97		5.83		0.86
				4.99		5.03		0.04
				4.76		5.94		1.18

between the amino-acid nitrogen of the filtrate before (Column B) and after (Column D) hydrolysis. The figures for total diamino nitrogen (Column F) represent the difference between the amino-acid nitrogen of the hydrolyzed filtrate before (Column D) and after (Column E) precipitation with phosphotungstic acid. The figures under Column H give the fraction of the peptide nitrogen which was present in the form of diamino-acids. The values are derived

from the difference between the total diamino nitrogen (Column F) and the free diamino nitrogen (Column C).

Small amounts of peptide nitrogen were found in most of the bloods examined. No significance is attached to figures less than 1.0 mg. as these probably fall within the limits of experimental error. The three normal human bloods examined showed less than 1.0 mg. of peptide nitrogen. Of five samples of beef blood, one showed a complete absence of peptide nitrogen, one less than 1.0 mg., and three less than 2.0 mg. per 100 cc. of blood. This forces the conclusion that none of these beef bloods contained significant quantities of peptide nitrogen. These results differ from the findings of Hiller and Van Slyke (9), who report an average of 7.0 mg. of peptide nitrogen per 100 cc. of beef blood. Their figures are based on analyses of filtrates obtained by precipitation of the blood with 2.5 per cent trichloroacetic acid.

Of the thirty-seven bloods from hospital patients, all but six showed more than 1.0 mg. of peptide nitrogen. The highest figure was 7.91 mg. One blood with a total non-protein nitrogen of 170.80 mg. contained practically no peptide nitrogen. Other bloods with a high total non-protein nitrogen contained an average of about 5.0 mg. of peptide nitrogen per 100 cc. of blood. In probably none of the bloods, however, was there enough peptide nitrogen to account for all of the undetermined nitrogen usually found in blood.

It should be pointed out that the procedure here used for the estimation of peptide nitrogen, depending on the determination of the amino-acid nitrogen by the Van Slyke method before and after hydrolysis of the mixed amino-acid and peptide solution, is not strictly accurate. The conjugated amino-acids of the blood probably occur in the form of the simplest peptides. The free amino groups of these bodies are readily deamidized with nitrous acid, thus increasing the value of the free amino-acid nitrogen and to that extent causing a negative error in the calculation of the peptide nitrogen.

We cannot support the conjecture of Folin and Berglund that the unknown nitrogen is partly due to histones. These bodies would yield, on hydrolysis, large increases of basic amino-acids.

With regard to the latter it will be recalled that neither the method of Van Slyke nor that of Folin does fully account for the

nitrogen of the hexone bases. The undetermined nitrogen from these blood constituents may account for a small fraction of the unknown nitrogen. That it is not due entirely to the presence of polypeptides may be also gathered from the figures of Leopold and Bernhard (1) and Woods (3) on the residual nitrogen in the alcoholic filtrates of the bloods they analyzed.

#### SUMMARY.

The presence of peptide nitrogen has been demonstrated in a number of bloods. No significant quantities of peptide nitrogen were found in three normal human bloods and five samples of fresh defibrinated beef blood. The amount of peptide nitrogen found in the bloods from hospital patients is by far too small to account for the undetermined nitrogen of pathological bloods with a high non-protein nitrogen. It would probably not fully account for the unknown nitrogen of bloods with a total non-protein nitrogen falling within the normal limits, only perhaps in exceptional cases.

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## THE PREPARATION OF CREATININE FROM CREATINE.

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### INTRODUCTION.

The usual methods for the preparation of creatinine have involved the use of urine as the original source. This has made its preparation rather laborious and its price almost prohibitive for any other purpose than small scale research. In view of the fact that creatine is now available in large quantity and at much reduced prices it has seemed desirable to consider methods for the preparation of creatinine from creatine, with the object of discovering a simple method, suitable for large or small scale operation, and involving relatively little time or expense.

The two chief methods already described for the preparation of creatinine from creatine are those of Folin and Denis (1), and of Benedict (2). The former involves the heating of solid creatine in an autoclave; the latter involves the intermediate preparation of creatinine zinc chloride by one of several methods, and the subsequent decomposition of this salt with ammonia. These methods do not seem as simple or as satisfactory for the purpose outlined above as the one described below.

### *General Method.*

The principle upon which the method depends is extremely simple, involving (*a*) the conversion of creatine into creatinine hydrochloride by treatment with hydrochloric acid, and (*b*) the formation of creatinine by treatment of the creatinine hydrochloride with ammonia, taking advantage of the facts that ammonia will liberate creatinine from solutions of its salts; that creatinine is only moderately soluble in cold concentrated ammonia; that ammonium chloride is readily soluble in cold ammonia

solutions; and that ammonia has very little tendency to bring about the conversion of creatinine into creatine.

The experimental technique necessary to obtain a good yield is described below. A number of variations in procedure are possible, and the choice of them may be determined somewhat by the scale of operation, the facilities of the particular laboratory, and the quality of the creatinine which may be desired.

#### EXPERIMENTAL TECHNIQUE.

The materials used were largely commercial creatine, containing 1 molecule of water of crystallization, together with small amounts of creatinine and traces of meat extractives. Pure recrystallized creatine (hydroxide) was also employed in certain cases.

Since the process divides clearly into two distinct steps, (1) the preparation of creatinine hydrochloride, and (2) the conversion of creatinine hydrochloride into creatinine, it seems best to consider these separately, particularly as the two may be combined in a number of different ways.

##### *Preparation of Creatinine Hydrochloride.*

(a). Creatine may be converted quantitatively into creatinine hydrochloride by evaporation to dryness upon a steam bath with an excess of hydrochloric acid, or by boiling such a solution slowly to dryness over a small flame. This is essentially the method employed by Benedict (3), who worked, however, only with relatively small quantities of creatine. We have found that complete conversion takes place when solid creatine is treated with twice its weight of 6 N hydrochloric acid, and the solution evaporated as indicated above. The process is rather a slow one when large quantities of creatine are employed, as a crust of creatinine hydrochloride forms when the solution becomes concentrated, and this must be broken up frequently. Furthermore, when crude creatine is thus treated considerable color develops, which increases somewhat with the time of heating. Material prepared by this process will be known as "creatinine hydrochloride (a)."

(b). Creatine may be converted into creatinine hydrochloride by treatment with *gaseous* HCl at room temperature. The

reaction is a slow one, but the process is practically automatic and has certain other advantages. In our experiments 100 gm. of creatine, previously dried at 100°C. to remove water of crystallization, were placed in a closed flask connected to an automatic HCl generator (*e.g.*, a Kipp generator employing fused NH<sub>4</sub>Cl and H<sub>2</sub>SO<sub>4</sub>). The HCl is absorbed rapidly at first, and in the course of a few hours the mixture has caked somewhat. Two or three times a day the flask is disconnected and the cakes are broken up with a glass rod. By the end of 48 hours the reaction is complete, as is evidenced by failure to absorb more HCl. The mixture will have become moist at the end of the reaction, because of the liberation of water in the reaction. The moist mass is dried for a short time at 100°C. It will be known as "creatinine hydrochloride (*b*)."

(c). Creatinine may be converted into creatinine hydrochloride solution by adding to it a very slight excess of hydrochloric acid and heating the mixture on the steam bath in a closed flask for a sufficient length of time. In our experiments 150 gm. of creatine (hydroxide), 85 cc. of concentrated HCl (sp.gr. 1.19), and 25 cc. of water were heated for 24 hours or more on the steam bath, when conversion was found to be complete. This solution will be known as "creatinine hydrochloride solution (*c*)."

#### *Conversion of Creatinine Hydrochloride into Creatinine.*

The conversion of creatinine hydrochloride into creatinine may also be accomplished in several ways.

(d). Solid creatinine hydrochloride is added to a volume of cold concentrated ammonia (sp.gr. 0.90) equal to the weight of creatinine hydrochloride used. (It may be noted that this is almost exactly equal to the weight of creatine hydroxide originally taken.) The mixture is stirred, lumps are broken up as thoroughly as possible, and after standing at 0°C. for an hour or so the creatinine is filtered off (with suction), washed with a little ice-cold concentrated ammonia, and finally with alcohol. It is dried at 100°C.

(e). Solid creatinine hydrochloride is dissolved in 0.8 of its weight of water, warming to effect solution, and ammonia gas is rapidly passed into the solution, the mixture being cooled in ice

at the same time. When the ice-cold mixture gives a *strong* odor of ammonia the current of gas is stopped and after standing an hour or so the creatinine is filtered off, washed, and dried as above.

(f). Solid creatinine hydrochloride is dissolved in 0.6 of its weight of water, heating to effect solution, and an equal volume of cold concentrated ammonia is added, with constant stirring. The mixture is cooled as rapidly as possible, and is allowed to stand at 0°C. for an hour or so, and is then filtered and treated as above.

(g). Concentrated creatinine hydrochloride solution (c) is treated with ammonia gas exactly as described in (f).

#### *Results.*

1. The *yield* of creatinine by the above methods may be summarized as follows. Combining (a) or (b) with (d) or (e) gives very uniformly a yield of about 90 per cent of the theory (69 to 70 per cent of the weight of creatine hydroxide used; theory 76 per cent). Combining (c) and (g) also gives about 90 per cent of the theory. Combining (a) or (b) with (f) gives 83 to 85 per cent of the theory, the lower yield being due to the larger volume employed.

2. The *purity* of the creatinine depends partly upon the purity of the original creatine and partly upon the process employed. Process (d) gives a product nearly always containing a small amount of chlorine, and while very convenient, is not recommended where a good grade of creatinine is desired. Process (e) gives a product which is practically 100 per cent pure, when tested by the Folin method of analysis against pure creatinine picrate (4) or against pure creatinine.<sup>1</sup> If *pure* creatine was used as a starting point the creatinine will be perfectly white; commercial creatine gives a faintly yellow product, the color depending upon the process employed for preparing the creatinine hydrochloride. Process (b) gives a whiter product than (a). The creatinine should give no test for chlorine with silver nitrate, or at most only a faint trace. Process (f) gives a product similar in every respect to that produced in (e). Process (g) gives a

<sup>1</sup> We are indebted to Mr. H. J. Bean for a sample of pure creatinine prepared especially for standardization purposes.

product similar in purity to that obtained by (e) or (f), but usually of lighter color than that produced by combining (a) with (e) or (f).

Attempts to remove the color entirely by decolorizing the creatinine hydrochloride solutions with charcoal were successful, but the yield was materially lowered thereby (to 80 per cent) as the charcoal adsorbs considerable creatinine from the very concentrated solution.

#### DISCUSSION.

Considerable quantities of creatinine have been prepared by the methods outlined above, as much as 1 kilo of creatine having been handled at one time, although most of the experiments employed 10, 25, 50, or 100 gm. All the methods seem suitable for large or small scale operation. It is believed that the small amount of attention required, the low cost of chemicals, and the high yield of creatinine obtained make these methods superior to any others hitherto proposed for the preparation of creatinine.

It should be noted that the methods are equally applicable to treatment of mixtures of creatine and creatinine, or to the repurification of creatinine.

The creatinine thus prepared should be of sufficient purity for any ordinary purpose (including Major's test for renal function (5)). It is not intended as an analytical standard, but it may be noted that in our experience it gives results undistinguishable from those obtained with samples prepared especially for this purpose.

#### *Recrystallization of Creatinine.*

The purification of creatinine by crystallization presents certain difficulties. If recrystallized from water at high temperatures or from aqueous alcohol, there is always the danger that some creatine may be formed, and this is almost impossible to separate by recrystallization, as it is much less soluble in all ordinary solvents than creatinine, and has a similar temperature coefficient of solubility. Crystallization from pure alcohol is satisfactory for a small sample, but the solubility is too small for convenient handling of large quantities of creatinine.

We have found the following procedure fairly satisfactory. 1 part by weight of creatinine is dissolved in 5 parts of water, previously heated to 65°C., as rapidly as possible. 2 volumes of acetone are added at once, and the mixture is cooled in ice. After standing a few hours the creatinine is filtered off, washed with acetone, and dried. About 65 per cent of the original creatinine is recovered. The solubility of creatinine in aqueous acetone is less than in aqueous alcohol of the same volume percentage, and the purity of the recrystallized material seems higher with acetone than with alcohol.

#### SUMMARY.

1. Simple methods have been described for the preparation of creatinine from creatine.
2. The yield and purity of the creatinine by these methods are most satisfactory.

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## DYE-PROTEIN AGGREGATES.

### I. CONGO FIBRIN.

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In a series of investigations, projected under the general title of "Dye-protein aggregates," an attack is being made upon the following questions: (1) What are the essential factors in the preparation of dyed proteins of uniform composition? (2) How is the proportion of dye in a given preparation affected by the dialyzability of the dye? (3) Do mass law concepts make possible a comprehensive description of digestion of dyed proteins by enzymes?

It is hoped that this work will contribute to the clarification of the subject of "adsorption compounds." For the present it has seemed advisable to speak of the dyed proteins as aggregates rather than as compounds.

In the present paper is described a special preparation of Congo fibrin, and some of the properties of this preparation are given together with some preliminary quantitative experiments on its digestion by pepsin HCl.

#### *Dyeing of Colloidally Dispersed Proteins.*

In criticizing various colorimetric methods by which the rate of digestion of proteins may be followed, Mathews<sup>1</sup> states: "In the first place it is impossible to get two pieces of fibrin of exactly the same surface of contact between it and the enzyme solution. And, in the second place, the combining power of the fibrin for Congo red or any other color, increases as digestion proceeds

<sup>1</sup> Mathews, A. P., *Physiological chemistry*, New York, 3rd edition, 1920, 360.

and more molecules are set free." Such a criticism may well apply to preparations of Congo fibrin made by staining finely hashed fibrin; for it is scarcely possible that such a typically colloidal dye as Congo red can reach an adsorption equilibrium with all fibrin "molecules" when the latter are as well aggregated as they must be in macroscopic particles of hashed fibrin.

It occurred to the writer, however, that it should be possible to make a quite homogeneous preparation of stained protein by staining the latter while in colloidal dispersion. Pursuant to this idea, a preparation of dyed protein was made in 1919 as follows: About 50 gm. of well washed and finely hashed fibrin from which the excess of water had been squeezed out, were dissolved in 500 cc. of hot 0.1 N NaOH. The solution was filtered through glass-wool, and in it were dissolved 5 gm. of Congo red. The whole formed a clear, deep red solution. The alkali protein and dye were then thrown down together by addition of N H<sub>2</sub>SO<sub>4</sub>. The dyed protein—hereafter called Congo fibrin—flocculated well, leaving a virtually colorless supernatant liquid. The Congo fibrin was then washed three times by decantation with distilled water. To render the preparation as insoluble as possible, it was suspended in 500 cc. of a saturated solution of NaCl, and boiled with constant stirring for 30 minutes. The whole was filtered through a Buchner funnel and washed repeatedly with large volumes of boiling distilled water until no further color was removed. The Congo fibrin was then dried *in vacuo* over sulfuric acid, pulverized, and passed through a 100 mesh sieve.

The following are some properties of this Congo fibrin. A suspension in glycerol has yielded no free Congo red to the glycerol in more than 2 years. A suspension in distilled water may be kept at 10°C. for a month without yielding up any color. A suspension in 0.1 N HCl requires several days at room temperature before any color is given up to the supernatant liquid. A suspension in 0.5 per cent sodium carbonate shows some swelling and a slight loss of color in a few hours at room temperature. The material is rapidly and completely digestible by pepsin HCl and by trypsin Na<sub>2</sub>CO<sub>3</sub>. The mean dimensions of the individual particles, which are quite irregular in shape, range from about 0.01 to 0.25 mm. Under considerable magnification the

particles, from the most minute to the largest, appear to be perfectly homogeneous in respect of the distribution of the Congo red. Colorimetric comparison of a completely digested sample with a sample of Grübler's Congo red shows the Congo fibrin to contain 7.2 per cent of Congo red.

A second and simpler method of preparing dyed protein than the one just described is the following: White of egg is strained through cheese-cloth, then diluted with 4 volumes of 5 per cent NaCl, and filtered through cotton- or glass-wool. An excess of the dye, previously brought into solution, is added to the egg white solution. The mixture is then heated in a boiling water bath with frequent stirring. The coagulated and dyed protein is thrown on a Buchner funnel and washed repeatedly with boiling distilled water until the filtrate is colorless. This washing is very tedious; for example, a mass of moist dyed protein of about 40 gm. in weight requires from 50 to 100 washings of 200 cc. each. The material is then preserved in glycerol or dried *in vacuo*.

#### *Size of Particles and Rate of Peptic Digestion.*

If an accurate colorimetric method for following the progress of digestion of proteins, or for the quantitative estimation of peptic activity is to be devised, it becomes necessary to determine how the rate of digestion of the dyed protein is related to the size of the particles being digested. An attempt was, therefore, made to limit the range of size of individual particles in the Congo fibrin by fractional centrifugation. Two lots of 15 cc. each of a well mixed suspension of the Congo fibrin in glycerol—each lot containing 0.4 gm. of powdered Congo fibrin—were centrifuged for 1 minute at 2,800 R.P.M. The supernatant material was carefully poured off, and the residues in the two tubes were united and the tubes rinsed out with glycerol until a total of 20 cc. was obtained. This suspension was labeled F1. The volume of the supernatant liquid in each tube was made up to 15 cc. with glycerol, thoroughly mixed, and centrifuged for 2 minutes at the same speed. The supernatant material was again removed and the residues of the two tubes were combined in a total of 20 cc. in the same manner as F1. The 2 minute residue was called F2. A similar method was used to obtain the remaining fractions. The supernatant material

from the previous centrifugation was centrifuged 4 minutes for F3; 8 minutes for F4; 16 minutes for F5; 32 minutes for F6; and 64 minutes for F7. The supernatant material from F7 was called F8.

This method of fractionation was not particularly successful in limiting the range of size of particles in each fraction; for the larger particles carried along with them large numbers of the smallest particles. Nevertheless, the results obtained in the peptic digestion of the several fractions appear to yield some definite information. The sizes of the individual particles were obtained as follows: A drop of the mixed glycerol suspension was mounted on a slide, set in a mechanical stage, and the vertical diameter of each particle as viewed through a low power, was obtained with an eyepiece micrometer. In this way, by measuring a sufficiently large number of particles, and by taking the apparent vertical dimension of each particle, irrespective of the position in which the latter might be lying, it was assumed that in the long run the mean dimension was being measured. Above 500 particles each of F2, F4, and F6 were measured. The mean dimensions, in millimeters, are given below.

F2.....	0.0767 ± 0.0017 mm.
F4.....	0.0508 ± 0.00095 "
F6.....	0.0215 ± 0.00030 "

It has already been mentioned that in the fractionation of the original glycerol suspension, the large particles carried down mechanically many of the smaller particles. This is further evidenced by a reduction in the variability of size of the particles as the mean dimension became smaller. The variability of the particles in F2 was 74 per cent, of F4 63 per cent, and of F6 47 per cent of their respective mean dimensions.

The yield of each fraction, in percentage of the original suspension, was obtained by the colorimetric comparison of completely digested aliquots of the fraction and of the original. The results are given in the fourth column of Table I. In the fifth column of this table are given the volumes of the aqueous suspensions of the respective fractions which contained equal masses of substrate. These volumes were measured out into tubes, centrifuged, and all but 5 cc. of the supernatant liquid of

each fraction discarded. These 5 cc. quantities, containing equal masses of substrate, were digested for 30 minutes with pepsin HCl in a manner to be described later. The percentage of digestion is given in the sixth column of Table I. The relative completeness of digestion reached by the first fractions is attributable to the large proportion of small particles in these fractions.

Preparations of each fraction were subjected to peptic digestion for different time periods, and the progress of the digestion was measured by the amount of Congo red set free. Since all but the smallest particles, when in suspension in water, settled rapidly, it was necessary to keep the suspension agitated during the digestion period. A special bath was devised in which 15 cc. centrifuge tubes, containing the digestion mixtures, could be submerged under water at 37°C., and rotated in the direction of their long axes at the rate of fifteen times per minute.

When hydrochloric acid is added to an aqueous suspension of the Congo fibrin, the particles turn blue, but do not lose any of the dye.<sup>2</sup> When pepsin is also present, the digestion of the protein is accompanied by an equivalent amount of dispersion of the dye. It has been found more satisfactory, however, in making the final color comparisons of the dye set free by digestion, to have the material at such a pH that the typical red color is developed. To obtain this result, an alkaline borate solution has been used as a buffer. This was made by dissolving 12.4 gm. of boric acid in 100 cc. of N NaOH and diluting with distilled water to 500 cc. instead of to a liter, as in the original Sörensen solution.<sup>3</sup> In addition to bringing each digest to the same pH (7.1 to 7.2), this buffer has the further value of stopping the peptic digestion practically instantly.

<sup>2</sup> A detailed study of this phenomenon, of significance in connection with recent theories respecting protein behavior, is reserved for the second paper of this series. Suffice it that aggregates of Congo red and of brom-phenol blue with the proteins of egg white have been prepared which, in aqueous suspension, can be carried through a range of pH from 2 to 8 and back again without loss of dye. The color of the suspended particles changes, however, with change of pH; and the alterations in color parallel those seen with solutions of the free dyes.

<sup>3</sup> Clark, W. M., *The determination of hydrogen ions*, Baltimore, 1920, 78.

## Dye-Protein Aggregates. I

TABLE I.  
*Fractionation of Glycerol Suspension of Congo Fibrin.*  
*Digestion of Fractions by Pepsin.*

Fraction.	Time of centrifugation.	Mean dimension of particles.	Fraction of original suspension.	Volume containing unit mass of Congo fibrin.	Amount digested by pepsin in 30 min.
	min.	mm.	per cent	cc.	per cent
F1	1		13.1	8.9	60
F2	2	0.077	21.6	5.4	62
F3	4		23.3	5.0	72
F4	8	0.051	15.7	7.4	78
F5	16		10.7	11.0	85
F6	32	0.021	5.0	24.5	92
F7	64		3.5	33.7	100
F8			8.3	14.0	100

TABLE II.  
*Peptic Digestion of Suspension of Congo Fibrin (O) and Its Fractions (F1 to F8).*

Standard conditions: 5 cc. suspension + 3 cc. 0.25 N HCl + 1 cc. pepsin (1:1,000).

Sus-pension.	O	F1	F2	F3	F4	F5	F6	F7	F8
Mass in 5cc., mg.	6.7	5.2	8.6	9.3	6.3	4.3	2.0	1.4	3.3
Time of digestion.									
Amount of digestion.									
min.	per cent								
4	2	0.0	0.0	0.0	0.0	0.0	0.0	4	21
8	8	7	3	4	4	6	3	14	40
12	35	20	8	15	12	15	15	32	73
16	52.5	34	20	28.5	25	36	38	62	93
20	65	46	32	43	40	64	66	85	100
24	75	58	40	59	55	80.5	83	96	
28	82	67.5	47.5	71	70	91	92.5	100	
32	87.5	75.5	61	82	84	96	98		
36	91	81	71	91	93	98	100		
40	94	85	74	96	98	100			
44	96	89	83	98	100				
48	98	92.5	89	100					
52	100	96	93						
56		98	96						
60		100	100						

The digestions were carried out as follows: Each fraction was made up to 100 cc. with distilled water. In each of a series of 15 cc. centrifuge tubes were pipetted 5 cc. of the well agitated suspension. To each tube were added 3 cc. of 0.25 N HCl and 1 cc. pepsin solution (Merck's, 1:1,000). Each tube was closed with a solid rubber stopper, and just at the moment that it was put in the bath, was well mixed. It may be added that digestion did not commence until the tube was put in the bath; for the particles formed a red residue at the bottom of each tube and did not change color until it was shaken. At the end of the digestion period for a given tube, it was removed from the bath, and 6 cc. of the alkaline borate solution were run in from a burette, and the whole was quickly mixed. In this way digestion in any tube could be stopped within 10 seconds. The pH at this point, determined by the hydrogen electrode, ranged between 7.1 and 7.2. Enough tubes were run so that in one or more digestion was complete. The amount of digestion in any tube was then obtained by colorimetric comparison, using the completely digested preparation as the standard. A simple dilution method of comparison was found sufficient, using the Myers colorimeter.

In Table II are given the results, expressed in percentages of complete digestion, obtained for the peptic digestion of the original glycerol suspension and the several fractions. In each series there is an initial stage in which the rate of digestion increases rather slowly, then an intermediate stage of rapid digestion and, finally, a terminal stage in which the rate of digestion diminishes. In the initial stage some time is apparently consumed in the attainment of some form of equilibrium between enzyme and substrate. When this occurs, etching away of the particles rapidly attains a maximum velocity, the smallest particles disappearing first. At the end of this stage of rapid digestion only the remains of the largest particles are to be found in a given digest. The terminal stage is, therefore, taken up by the relatively slow digestion of these largest particles.

When the percentages of digestion for a given series are plotted against the corresponding digestion times, an S-shaped curve is obtained. In the case of the middle fractions, F<sub>4</sub> and F<sub>5</sub>, which were practically free from the largest and smallest particles, these curves are fairly smooth. To one, who has a penchant

for the mass law interpretation of biochemical phenomena, this form of curve immediately suggests an autocatalytic reaction. When it is noted, however, that in the case of F4 only 6.3 mg. of the Congo fibrin were suspended in 9 cc. of liquid in each digest—a concentration of 0.07 per cent—it is extremely doubtful whether the concentration of the products of digestion would be sufficient either to accelerate the reaction or to inhibit it. The writer prefers to interpret these curves as expressing, in integral form, the frequency distribution of amounts of digestion occurring in the Congo fibrin particles. Where the curve is symmetrical with respect to the mean, the velocity of digestion, between the limits of 25 and 75 per cent, will oscillate about a constant value. The results with F4 indicate that the experimental conditions can be controlled so that symmetrical curves for the progress of peptic digestion can be obtained. Part of the work still in progress is concerned with the preparation and standardization of dyed proteins suitable for precise measurements of peptic activity.

#### SUMMARY.

1. Methods for the preparation of homogeneous aggregates of dye and protein are described.
2. Results of peptic digestion of Congo fibrin suspensions show that the progress of digestion is largely dependent upon the size of the particles of substrate.
3. Peptic digestion of the Congo fibrin can be followed accurately with quantities weighing less than 10 mg., and in a concentration of the substrate such that the products of digestion can exert no appreciable effect upon the reaction.
4. It is questionable whether mass law concepts can be applied to the description of the digestion of fine suspensions of Congo fibrin by pepsin.

## SOME OBSERVATIONS IN REGARD TO GROWTH-PROMOTING SUBSTANCES OF BACTERIAL ORIGIN.

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### INTRODUCTION.

From time to time papers have appeared in which growth-promoting substances in cultures of bacteria have apparently been demonstrated. Thus, Pacini and Russell (1) reported the presence of such a substance in extracts of cultures of *Bacillus typhosus* fed to white rats. This, as far as the writer is aware, is the only experimental work on record in which cultures of bacteria or extracts of cultures have been successfully used to stimulate growth in rats. Damon (2) attempted to verify this observation, but was unsuccessful with cultures of *Bacillus paratyphosus B*, *Bacillus coli*, and *Bacillus subtilis*. However, numerous investigators have successfully used cultures of bacteria or extracts of bacteria to stimulate the growth of other microorganisms. Cantani (3) reported more profuse growth of *Bacillus influenzae* in symbiosis with *Bacillus diphtheriae*, *Micrococcus gonorrhæae*, and certain staphylococci than he was able to get when growing this organism in pure culture. He also observed a stimulation of growth when the agar on which *Bacillus influenzae* was grown was enriched by the addition of emulsions of dead bacteria. This growth he believed to be induced by some factor contained within the dead cells. Neisser (4) reports successful growth of *Bacillus influenzae* in symbiosis with *xerosis bacilli*, but was unsuccessful in his attempts when the medium was enriched with killed cultures of this organism. Bottomley (5) observed the elaboration of a stimulating substance in *Sphagnum* peat by the growth of certain aerobic soil bacteria. Its presence in this case was indicated by a relatively accelerated fixation of nitrogen by *Azobacter*, when

grown in such "bacterized peat," over the normal rate of fixation in "unbacterized" peat. And finally, Thjöftta (6) was able to grow *Bacillus influenzae* in plain beef extract-peptone broth when killed emulsions of Friedlander's bacillus and the ozena bacillus were added to it. He was also successful when aqueous extracts of these organisms or of *Bacillus proteus* were used. Incidentally, it may be mentioned that the writer (7) was unable to confirm these findings.

The results embodied in this report have been obtained in a series of experiments designed to confirm the findings of Bottomley and Thjöftta and to extend somewhat further the investigation of growth-promoting substances of bacterial origin. Too frequently, we believe, these substances have been termed vitamins, or it has been suggested that they might be related to vitamin B in that they induced accelerated growth on the part of the test organism. To us it would seem that all these observations were open to criticism, as has been intimated by Meader and Robinson (8), because the biological test, *i.e.* on young rats, is the only unobjectionable test that can be used to demonstrate the presence or absence of vitamin. The experimental work herein reported gives the results of such a test.

#### EXPERIMENTAL.

In all of the experiments young albino rats of carefully selected stock were used. During the preliminary period of the first experiment these animals were fed a ration previously demonstrated to be adequate in all the food essentials except vitamin B. This ration had the following composition:

	gm.
Casein (purified).....	18.0
Starch.....	42.5
Cane-sugar.....	17.0
Lard.....	15.0
Butter fat.....	5.0
Salt mixture 185 (9).....	2.5

On this diet young growing rats maintain their body weight for about 2 weeks and then begin to lose weight rapidly unless the deficiency of vitamin B is satisfied. It is during this period of decline that the substance, the vitamin content of which it is

desired to test, is added. The presence of vitamin will be noted in the upward trend of the weight curve.

The bacteria that were chosen for testing for their content of vitamin B were selected as representing (a) the group of aerobic spore formers, (b) the mucoid organisms of the *Bacillus mucosus capsulatus* group, and (c) the acid-fast bacteria. These organisms were found especially adapted for use in this work, as it was comparatively easy to grow and desiccate them in sufficient quantity to provide adequate material for feeding experiments.

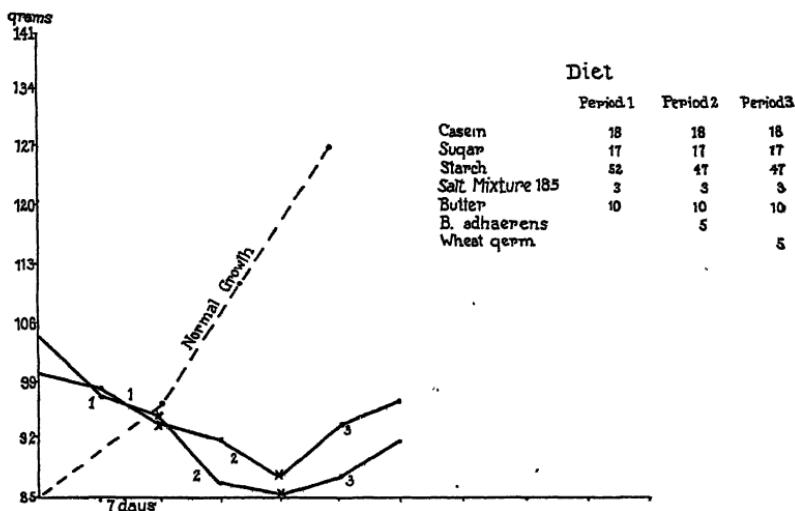


FIG. 1. During Period 1 the diet was adequate except in vitamin B. During Period 2, 5 per cent of *Bacillus adhaerens* replaced an equivalent amount of starch. It will be noted that the loss of weight was not checked. During the third period a return to normal growth was induced by adding 5 per cent of wheat germ in place of *Bacillus adhaerens*.

*Experiment 1.*—In this experiment it was thought advisable to test again the organic constituents of the culture medium, *i.e.* peptone and beef extract, for their content of vitamin B. Accordingly, these substances were substituted for an equivalent amount (7.5 per cent) of starch in the basal ration. Young rats fed on these mixtures failed to grow, and weight curves, indicating a continuous decline, that were essentially identical with those obtained in the original test (10), resulted. These were

interpreted as indicating that peptone and beef extract were devoid of any significant amount of the water-soluble vitamin.

*Experiment 2.*—In this test *Bacillus adhaerens*—an aerobic, spore-forming soil organism—was grown in bottles containing nutrient broth. Quart bottles were found to be especially useful because they offered a large surface area for the formation of a pellicle. After incubation at 37°C. and the formation of the pellicle, the organisms were filtered off by using ordinary filter paper, spread on a tray, and dried in the incubator at body temperature for 3 or 4 days. At the end of this time they had lost a large amount of moisture and decreased in bulk so they could be triturated in a mortar and desiccated. They were then added to the basal ration, replacing 5 per cent of the starch. The results of this feeding test are shown in the weight curves of two typical rats in Fig. 1. From these curves it would seem that no benefit was derived by the animals from the addition of the bacteria, but that they returned to a normal rate of growth during the third period of the experiment when wheat germ middlings, as a source of vitamin B, replaced the organisms.

*Experiment 3.*—The two organisms chosen as representing the mucoid group of bacteria were Friedländer's bacillus<sup>1</sup> and Pfeiffer's bacillus. They were grown on Prazmowski's medium in Kolle flasks with 1 per cent of the three sugars, dextrose, lactose, and saccharose, added. This medium has the following composition:

	gm.
Dipotassium phosphate.....	5.0
Magnesium sulfate.....	5.0
Ammonium carbonate.....	5.0
Calcium chloride.....	0.5
Water.....	1,000 cc.

Profuse growth was always obtained after 48 hours incubation at 37°C. This was removed with a sterile right-angled glass rod, dried, desiccated, and added to the ration in place of 5 per cent of starch. The diets and results of the feeding tests are shown in Figs. 2 and 3. It will be noted from Fig. 2 that the animals continued to lose during the second period; *i.e.*, while Friedländer's bacillus was added to the ration. In the case of Pfeiffer's

<sup>1</sup> The culture here referred to is a mucoid organism carried in this laboratory under the name Friedländer's bacillus, but fermenting lactose.

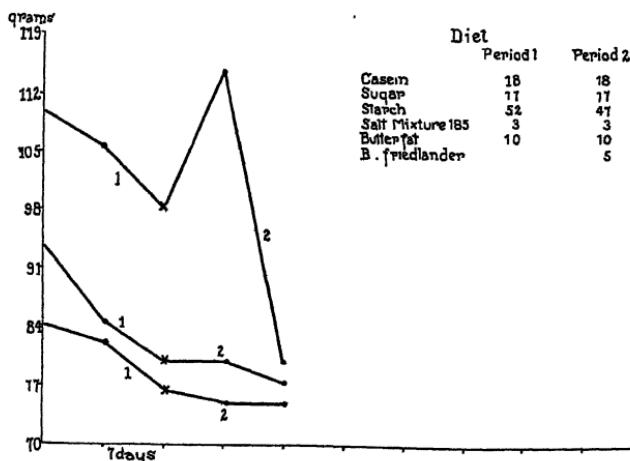


FIG. 2. During the second period 5 per cent of Friedländer's bacillus replaced an equivalent amount of starch. The curves would not seem to indicate the presence of any vitamin in the bacteria.

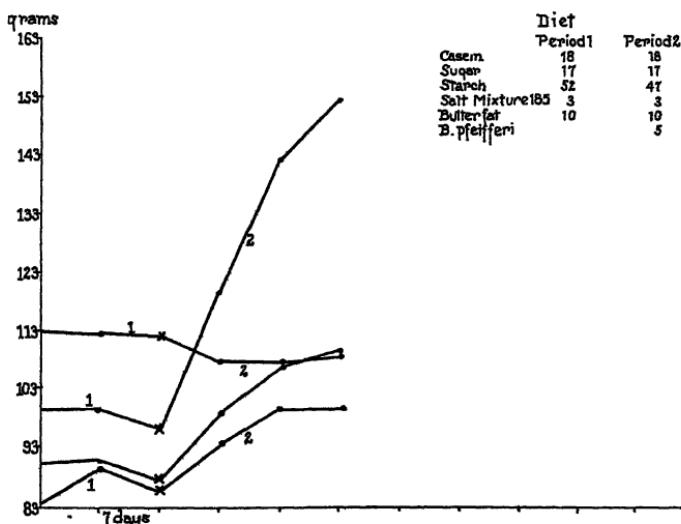


FIG. 3. Pfeiffer's bacillus added in the amount of 5 per cent to the ration seems to supply enough of the growth-stimulating factor either to maintain the experimental animals at a constant weight or to cause them to gain weight rapidly.

bacillus, however, three of the four test animals exhibited a distinct gain in weight, as indicated in Fig. 3. Why this organism, so similar to Friedländer's bacillus, should give such a different result we do not attempt to explain at this time.

*Experiment 4.*—*Bacillus timothy 213*,<sup>2</sup> an acid-fast organism, was selected for testing as the representative of this group of bacteria. It was found to produce an abundant pellicle, when cultivated at 37°C., on veal infusion 4 per cent glycerol broth, and this medium

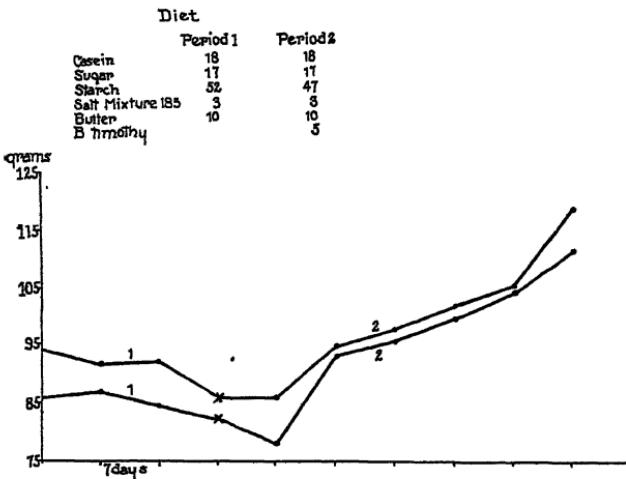


FIG. 4. 5 per cent of *Bacillus timothy 213* was added at the point marked by the cross. It will be seen that there is a notable resumption of growth in this case.

was used exclusively in this experiment. The formation of the pellicle in this case was relatively slow, and it was never possible to filter it off in less than 10 to 12 days. The treatment of the organisms subsequent to filtration was the same in this case as in all the other tests. Two feeding experiments were carried out however, and the results are shown in Figs. 4 and 5. In one case 5 per cent of the organisms was added to the ration and in the other 10 per cent was used to replace a like amount of starch

<sup>2</sup> The culture of *Bacillus timothy* here used is identical with *Bacterium phlei* or the timothy grass bacillus of Moeller and is carried in this laboratory as *Bacillus timothy 213*.

Examination of the growth curves seems to indicate the presence in these organisms of a growth-promoting substance that is capable of inducing rapid development of the animals and growth seems to be somewhat more rapid in the case of the addition of 10 per cent of the bacteria.

#### DISCUSSION.

The author desires to take strong exception to the use of the term "vitamin," as applied to any growth-stimulating substance of bacterial origin until such substance has been subjected to the

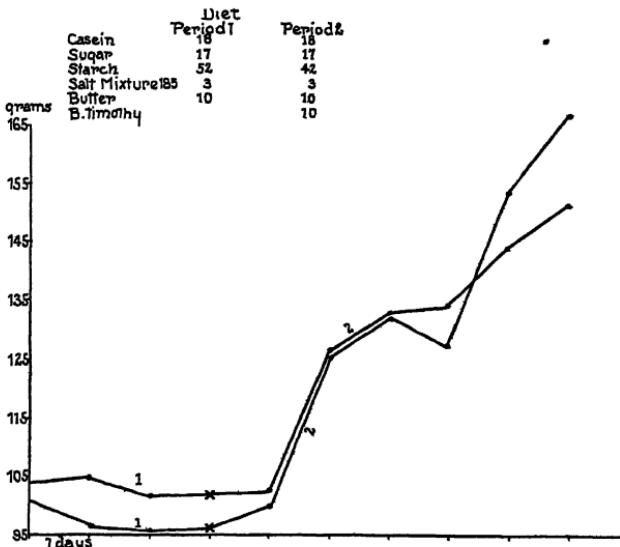


FIG. 5. 10 per cent of *Bacillus timothy* 213 added at the point indicated by the cross, seemed to stimulate the animals to practically normal growth.

biological test. In the work reported in this paper certain organisms to which growth-stimulating properties have been attributed have been subjected to such a test with widely divergent results. In the case of Friedländer's bacillus and Pfeiffer's bacillus, both members of the same group of organisms, no explanation of the discordant results presents itself. Up to this point the evidence for an accessory substance in bacteria would seem to be negative and it is not until we examine the results of feeding experiments with *Bacillus timothy* 213 that there appears any suggestive evidence on the other side of the question. In

this experiment there really appears to have been a substance supplied by the bacteria that satisfied the deficiency of vitamin B in the diet. We realize, however, that even this result may be open to question, because of the fact that these bacteria were grown on a veal infusion broth. The possibility that there was enough vitamin in such a broth that might have been adsorbed or absorbed by the bacteria and carried over in this way would appear to be remote, but should be borne in mind. To clear up such a point as this would necessitate cultivation of the bacteria in a medium every factor of which had been proven to be vitamin-free by feeding tests. Such tests are being carried on in this laboratory and will be reported on in a subsequent paper.

#### CONCLUSIONS.

1. Commercial peptone and beef extract have been again found to be devoid of vitamin B.
2. 5 per cent of a spore-forming aerobic organism, *Bacillus adhærens*, did not supply the deficiency of vitamin B in an otherwise adequate diet.
3. 5 per cent of a mucoid organism, Friedländer's bacillus, failed to induce growth in young rats.
4. 5 per cent of another mucoid organism, Pfeiffer's bacillus added to a diet deficient in vitamin B maintained the animals at a constant weight or induced rapid growth.
5. 5 per cent of an acid-fast organism, *Bacillus timothy*, had the same action as Pfeiffer's bacillus.
6. 10 per cent of *Bacillus timothy* induced rapid and continuous growth in rats limited to a diet deficient in the water-soluble vitamin.

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## STUDIES ON ENZYME ACTION.

### XXIV. THE KINETICS OF THE ESTER-HYDROLYZING ACTIONS OF SOME TISSUE AND TUMOR EXTRACTS.

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#### INTRODUCTION.

In connection with the study of the lipolytic actions of various tissues and tumors, it was necessary to obtain data relative to the kinetics of these actions, as well as of the effects of different concentrations of enzyme material and of substrate. The bearing of hydrogen ion concentration upon the kinetics of the actions to be described, connects this paper with a previous paper of this series (1) in which the results obtained in the study of the effects of the hydrogen ion concentrations were presented in some detail.

The results on the kinetics of the lipase actions to be given will be interpreted in terms of certain theoretical relationships which have been developed by others. The principal object of this paper is to compare these enzyme actions with other enzyme actions, and to furnish a foundation for presenting at a later date a comparative study of lipase, protease, and other enzyme actions of tumors and tissues under definite conditions which will permit of a certain degree of satisfactory comparison because of these preliminary data.

#### THEORETICAL.

The application of kinetic equations to the chemical changes brought about by enzymes has not yielded the results hoped for at various times. In the first place, it may be pointed out that the monomolecular reaction velocity equation only reproduces the rate of change in the substrate in enzyme actions in isolated

cases. A number of empirical equations have been proposed to reproduce the results, the most successful of the simpler ones being that of Schütz (2). One of the most recent developments attempts to account for the deviations from the simple monomolecular reaction velocity law on the basis of the occurrence of additional reactions in the medium, such as reaction between enzyme and products of enzyme action, accompanied by change in concentration of enzyme, etc. The equations developed by Northrop (3) include certain of these factors, and also show the theoretical significance of Schütz's equation (previously also explained by Arrhenius (4) on analogous grounds) as derivable from his, if certain limiting conditions are introduced.

The experimental results to be given here will be interpreted on the bases of the three equations: Monomolecular reaction velocity equation; Schütz's equation; and Northrop's equation. The first has been applied in the past to a large number of enzyme actions; the second, mainly to protease and lipase actions; and the third, practically only to protease actions.

#### EXPERIMENTAL RESULTS.

The enzyme material was obtained as described in a previous paper. The study was limited to the extracts of the Flexner-Jobling rat carcinoma (Experiments 45, 46, 47-Tu, 49, and 59) and of the rat leg muscle (Experiments 47-M and 50). These extracts were prepared with water as described previously (1) and filtered through paper.

Most of the actions were tested on glyceryl triacetate as perhaps the most satisfactory of the comparatively simple esters related to the fats. Some series were carried out also with phenyl acetate and with a number of additional esters. The use of glyceryl triacetate might perhaps be considered to introduce a complicating factor because of the polyacid nature of the alcohol. However, the reaction was not allowed to proceed far in most cases so that at most, the chemical change or hydrolysis was doubtless limited to the saponification of one acid equivalent in each molecule of ester.

The mixtures were brought to the desired hydrogen ion concentrations with sodium hydroxide or hydrochloric acid and tested colorimetrically, the indicators and standard solutions

described by Clark (5) being used. The reactions occurred at 37-38°, toluene being present throughout. The necessary blanks were run in every case. The actions are given in every case as cubic centimeters of 0.1 N sodium hydroxide solution used with phenolphthalein as indicator, corrected for blanks, or in other words, as tenths of milli-equivalents of ester hydrolyzed by the enzyme in the mixture titrated.

The first series of results to be presented shows the increases in actions on glyceryl triacetate with time. The experimental data are given in Table I. Two sets of the mixtures were made up for each series and measured portions titrated at the various time intervals. The titrated portions consisted of 5 cc. of the enzyme extract made up to 15 cc. and ester added. Table II shows the relative contents of the enzyme (*E*), the concentration of glyceryl triacetate (*A*) in the titrated portions in tenths of milli-equivalents, and the pH values at the start and when tested after certain time intervals. The mixtures contained the following parts of extracted material:

Experiment 45(A).....	15.0 mg. tumor tissue per cc. of mixture tested.
" 45(B).....	45.0 "
" 46(A).....	68.8 "
" 46(B).....	206.4 "
" 47(A-Tu)....	31.4 "
" 47(B-Tu)....	94.2 "
" 47(B-M)....	102.6 " muscle

It is evident that although the absolute actions were decreasing with the successive time intervals, they had not become zero. In order to study the kinetic relationships more satisfactorily, the results were plotted as shown in Figs. 1 and 2. The actions at definite time intervals taken from these curves are shown in Table III, and were used in the subsequent calculations. The pH values of the mixtures initially 7.0 had all become 5.0 to 5.4 at or soon after the measurements at the first elapsed time interval (ten  $\frac{1}{2}$  hour units) and did not change thereafter.

In Table IV are given the values of *K* as calculated according to the monomolecular reaction velocity equation  $K = \frac{1}{T} \log \frac{A}{A - x}$  from the results of Table III. These values decreased steadily for Experiments 45 and 46, and decreased for Experiments 47

## Kinetics of Lipase Actions

TABLE I.  
*Observed Rates of Actions on Glyceryl Triacetate.*

Experiment 45.			Experiment 46.			Experiment 47.		
Time in $\frac{1}{2}$ hr. units. "	Actions in tenths of milli- equivalents of ester hydrolyzed.		Time in $\frac{1}{2}$ hr. units.	Actions in tenths of milli- equivalents of ester hydrolyzed.		Time in $\frac{1}{2}$ hr. units.	Actions in tenths of milli-equivalents of ester hydrolyzed.	
	(A)	(B)		(A)	(B)		(A)	(B)
2	0	0	2	0.02	0.22	4	0.05	0.12
7	0.04	0.10	8	0.12	0.46	8	0.09	0.39
16	0.07	0.30	18	0.26	0.90	16	0.30	0.83
34	0.16	0.42	34	0.39	1.10	24	0.46	1.25
53	0.18	0.60	41	0.40	1.28	40	0.71	1.95
72	0.23	0.70	58	0.51	1.55	96	1.58	3.98
90	0.29	0.81	88	0.65	1.79	120	1.82	4.56
108	0.34	0.84	91	0.65	1.87	192	2.51	6.05
145	0.39	1.10	121	0.76	2.18	434	2.90	9.39
156	0.39	1.11	151	0.87	2.35			
179	0.39	1.21	184	0.93	2.58			
181	0.39	1.21	261	1.10	2.98			
192	0.41	1.23	294	1.19	3.20			
215	0.49	1.32	362	1.28	3.39			
268	0.55	1.58	395	1.37	3.60			
304	0.57	1.61	534	1.59	3.90			
345	0.69	1.79	567	1.61	4.18			
433	0.77	2.05						
533	0.89	2.26						

TABLE II.  
*Enzyme (E) and Substrate (A) Concentrations for Actions Given in Table I.*

Experiment No.	E (arbitrary units).	A (in tenths of milli- equivalents of glyceryl triacetate).	Initial pH.	Time in hrs. to reach pH 5.0 to 5.4.
45(A)	0.33*	20.4	7.0	4
45(B)	1.00*	34.0	7.0	4
46(A)	0.33†	20.4	7.0	14 (not tested before).
46(B)	1.00†	34.0	7.0	14 " " "
47(A-Tu)	0.33‡	20.4	5.2	
47(B-Tu)	1.00‡	34.0	5.2	
47(B-M)	1.00	34.0	5.2	

\* Concentrations comparable.

† Concentrations comparable.

‡ Concentrations comparable.

(B-Tu), (A-Tu), and (B-M) after an initial constancy extending over about 40 time units. The most obvious explanation of this decrease, assuming the kinetic equation to interpret correctly the chemical equation for each experiment, is a progressive decrease in enzyme concentration due to spontaneous inactivation or to

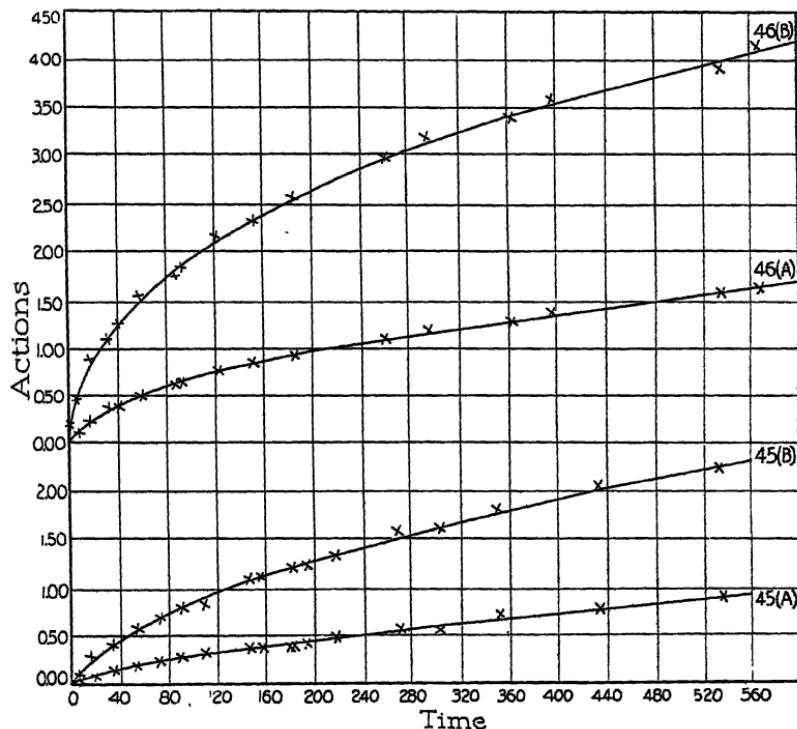


FIG. 1. Time-action results of two rat tumor extracts, Nos. 45 and 46, on glyceryl triacetate. Abscissa values,  $\frac{1}{2}$  hour units; ordinate values, tenths of milli-equivalents of ester hydrolyzed. Concentration of tumor, three times as great in the B series as in the A series; concentration of ester,  $1\frac{1}{2}$  times as great in the B series as in the A series.

the action of the products of the reaction. The initial constancy of  $K$  in Experiments 47 (B-Tu), (A-Tu), and (B-M) where the hydrogen ion concentration did not change, followed by the falling off in  $K$  indicates that the increasing concentration of the products of the reaction caused the decrease in  $K$ . The continuous

decrease of  $K_s$  in Experiments 45 and 46 may then be ascribed to a change in hydrogen ion concentration from pH 7.0 to 5.0 with the accompanying less favorable conditions for actions (1) during the first period, followed then by the increasing concentrations of the reaction products.

The values of  $K_s$  of Schütz's equation  $K_s = \frac{x}{\sqrt{T}}$  are given in Table V. The substrate was present in great excess in every case except Experiment 47 (B) where approximately 35 per cent had

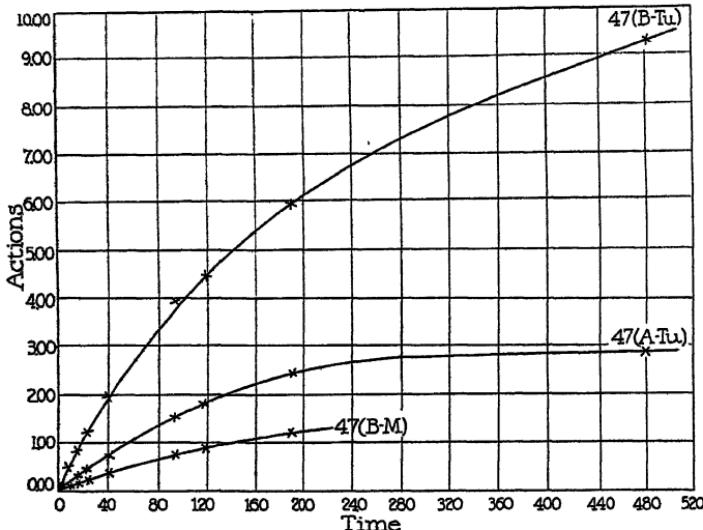


FIG. 2. Time-action results of rat tumor extract, No. 47-Tu, and rat leg muscle extract, No. 47-M, on glyceryl triacetate. Abscissa values,  $\frac{1}{2}$  hour units; ordinate values, tenths of milli-equivalents of ester hydrolyzed. Concentration of tumor, three times as great in the B series as in the A series; concentration of ester,  $1\frac{1}{2}$  times as great in the B series as in the A series.

reacted. The values of  $K_s$  in any one series first increased, then became constant, and in two series showed definite decreases. These results are similar to the results found by others for protease actions and for certain lipase actions. Also, the protease actions of some tumor and tissue preparations reported in a previous paper showed the same relations. It was shown by Northrop and others that the increase in  $K_s$  was due to insufficiency of reac-

TABLE III.  
*Lipolytic Actions After Definite Time Intervals from Figs. 1 and 2.*

Time in ½ hr. units.	Experiment No.						
	45(A)	45(B)	46(A)	46(B)	47(A-Tu)	47(B-Tu)	47(B-M)
10	0.05	0.17	0.14	0.55	0.18	0.50	0.09
20	0.09	0.27	0.25	0.85	0.35	1.00	0.18
40	0.15	0.46	0.42	1.25	0.70	1.95	0.34
60	0.21	0.61	0.53	1.52	1.03	2.72	0.48
100	0.30	0.87	0.69	1.93	1.60	4.02	0.77
150	0.38	1.09	0.84	2.32	2.15	5.23	1.05
200	0.45	1.28	0.98	2.64	2.53	6.20	1.30
250	0.51	1.46	1.08	2.98	2.76	6.96	
300	0.58	1.63	1.17	3.18	2.88	7.60	
350	0.64	1.78	1.27	3.39		8.16	
400	0.70	1.93	1.35	3.56		8.65	
450	0.76	2.07	1.44	3.73		9.08	
500	0.82	2.20	1.53	3.90		9.48	

TABLE IV.  
*Constants  $\times 10^4$  ( $K$ ) of the Monomolecular Reaction Velocity Equation Calculated from the Results in Table III.*

Time in ½ hr. units.	Experiment No.						
	45(A)	45(B)	46(A)	46(B)	47(A-Tu)	47(B-Tu)	47(B-M)
10	2.5	5.0	6.9	16.3	8.9	14.8	2.7
20	2.2	4.0	6.2	12.7	8.7	14.9	2.7
40	1.9	3.4	5.2	9.4	8.7	14.8	2.5
60	1.7	3.0	4.4	7.6	8.6	13.9	2.4
100	1.5	2.6	3.4	5.8	8.2	12.6	2.3
150	1.3	2.2	2.8	4.7	7.4	11.1	2.1
200	1.1	1.9	2.5	4.0	6.6	10.1	2.0
250	1.0	1.8	2.2	3.6	6.1	9.2	
300	0.96	1.6	2.0	3.3		8.4	
350	0.91	1.5	1.8	3.0		7.8	
400	0.87	1.5	1.7	2.8		7.3	
450	0.84	1.4	1.6	2.6		6.9	
500	0.81	1.3	1.6	2.5		6.5	

tion products; that when the products had reached a certain concentration, and the velocity of the reaction was inversely proportional to the concentration of the products,  $K_s$  remained constant; but that when the change in substrate concentration was large, the value of  $K_s$  decreased since Schütz's equation contains no term to include such a change.

The results calculated according to Northrop's equation

$$K_n = \frac{A \log_e \frac{A}{A-x} - x}{ET}, E \text{ (enzyme concentration) being assumed}$$

constant, are given in Table VI. Following initial irregularities or increases, constant values over greater or smaller ranges are obtained in most of the experiments, followed in some cases by decreases. The constancies of  $K_n$  do not appear to be more satisfactory than the values of  $K_s$  of Schütz's equation, but the theoretical bases are more satisfactory. The initial irregularities in Experiments 45 and 46 may have been due to a great extent, as already stated, to changes in hydrogen ion concentrations.

TABLE V.

*Constants  $\times 10^2$  ( $K_s$ ) of Schütz's Equation Calculated from the Results in Table III.*

Time in ½ hr. units.	Experiment No.						
	45(A)	45(B)	46(A)	46(B)	47(A-Tu)	47(B-Tu)	47(B-M)
10	1.6	5.4	4.4	17.4	5.7	15.8	2.9
20	2.0	6.2	5.6	19.0	7.8	22.4	4.0
40	2.4	7.3	6.6	19.8	11.1	30.8	5.4
60	2.7	7.9	6.8	19.6	13.3	35.1	6.2
100	3.0	8.7	6.9	19.3	16.0	40.2	7.7
150	3.1	8.9	6.9	18.9	17.6	42.7	8.6
200	3.2	9.1	6.9	18.7	17.9	43.9	9.2
250	3.2	9.2	6.8	18.7	18.2	44.0	
300	3.3	9.4	6.8	18.4		43.9	
350	3.4	9.5	6.8	18.1		43.6	
400	3.5	9.7	6.8	17.8		43.3	
450	3.6	9.8	6.8	17.6		42.8	
500	3.7	9.8	6.8	17.4		42.4	

In general, it may be said that these lipase actions agree with the kinetics of other enzyme actions heretofore described, and especially with the protease actions of similar preparations already given.

In addition to following the kinetics of the lipase actions over extended time periods, the results presented also permit of a direct comparison of the different concentrations of the same extract. This can be done most satisfactorily by comparing the

TABLE VI.

*Constants  $\times 10^5$  ( $K_n$ ) of Northrop's Equation Calculated from the Results in Table III.*

Time in hr. units.	Experiment No.						
	45(A)	45(B)	46(A)	46(B)	47(A-Tu)	47(B-Tu)	47(B-M)
10	10	0	1.5	45	7.4	39	1.0
20	0	10.0	7.7	54	16	76	2.7
40	0	6.0	10.8	61	31	146	5.1
60	3.7	9.0	11.9	59	45	192	5.8
100	1.9	11.6	12.2	57	66	257	9.3
150	2.3	12.3	11.9	55	81	299	11.1
200	2.5	12.5	12.1	54	87	323	12.8
250	2.4	13.4	11.9	55	90	331	
300	2.8	14.3	11.6	53		334	
350	3.1	13.9	11.8	52		335	
400	3.0	14.0	11.7	50		338	
450	3.4	14.5	11.9	49		330	
500	3.2	14.9	12.1	49		327	

TABLE VII.

*Times Required for the Same Actions with Different Enzyme Concentrations from the Results of Table III.*

Actions in tenths of milli- equiva- lents.	Experiment No.	$E$ Relative enzyme con- cen- tra- tion.	$T$ Time for action.	ET	Experiment No.	$E$ Relative enzyme con- cen- tra- tion.	$T$ Time for action.	ET
0.50	45(A)	0.33	234	78	45(B)	1.00	44	44
0.50	46(A)	0.33	54	18	46(B)	1.00	8	8
1.00	46(A)	0.33	208	69	46(B)	1.00	35	35
1.50	46(A)	0.33	480	160	46(B)	1.00	58	58
0.50	47(A-Tu)	0.33	28	9.3	47(B-Tu)	1.00	10	10
1.00	47(A-Tu)	0.33	56	18.7	47(B-Tu)	1.00	19	19
1.50	47(A-Tu)	0.33	92	30.7	47(B-Tu)	1.00	30	30
2.00	47(A-Tu)	0.33	136	45.3	47(B-Tu)	1.00	42	42
2.50	47(A-Tu)	0.33	192	64	47(B-Tu)	1.00	54	54

times required for the same absolute change with the same enzyme preparation at the different concentrations. The data from the curves are given in Table VII.

As pointed out by Arrhenius (6), for a given enzyme preparation, the product  $ET$  should be constant,  $T$  representing the time for a definite amount of change to take place, whether or not the monomolecular reaction velocity equation represents the rate of change. Table VII shows such a constancy only for Experiments 47(A) and 47(B) for four of the five sets of values. In considering the experiments, however, it must be recalled that for Experiments 45 and 46, the mixtures initially were at pH 7.0 and became constant at about pH 5.2 only after a few hours, while the mixtures in Experiment 47 were at pH 5.2 throughout. The first three values of  $ET$  for Experiment 47 (A-Tu) and the first four for Experiment 47 (B-Tu) show proportionate increases for the total absolute actions and the products  $ET$ . This indicates that the conditions of the enzyme action remained unchanged for these periods of time or of extents of reaction, both as regards the character of the enzyme and the character of the substrate, and also that the substrate was present in great excess.

In order to attempt to follow the kinetics of the actions starting at a number of different hydrogen ion concentrations, the results of two series, one with rat tumor tissue (Experiment 49, 39.3 mg. per cc. of mixture tested), and one with rat leg muscle (Experiment 50, 38.3 mg. per cc. of mixture tested) are shown in Table VIII and Figs. 3 and 4. The mixtures were brought to the different hydrogen ion concentrations indicated in the headings of the columns, and tested on glyceryl triacetate, 3.4 milli-equivalents per 15 cc. of the titrated portions. The results were presented in part in the previous paper on the effect of hydrogen ion concentration on lipase actions. The mixtures which were started at pH 4.0 remained at pH 4.0 throughout. For the tumor extracts (Experiment 49) even in  $2\frac{1}{2}$  hours, the mixtures had all reached values between pH 5.0 and 5.5, where they remained constant. The same was true for the muscle extracts except for the six results where the pH values found are shown in parentheses following the actions found.

The times required for the same successive total actions taken from the curves are shown in Table IX. The relative enzyme contents for the successive time intervals, calculated on the assumption of the constancy of the product  $ET$  and calling the enzyme concentration  $E$  one hundred at pH 9.0, are shown in

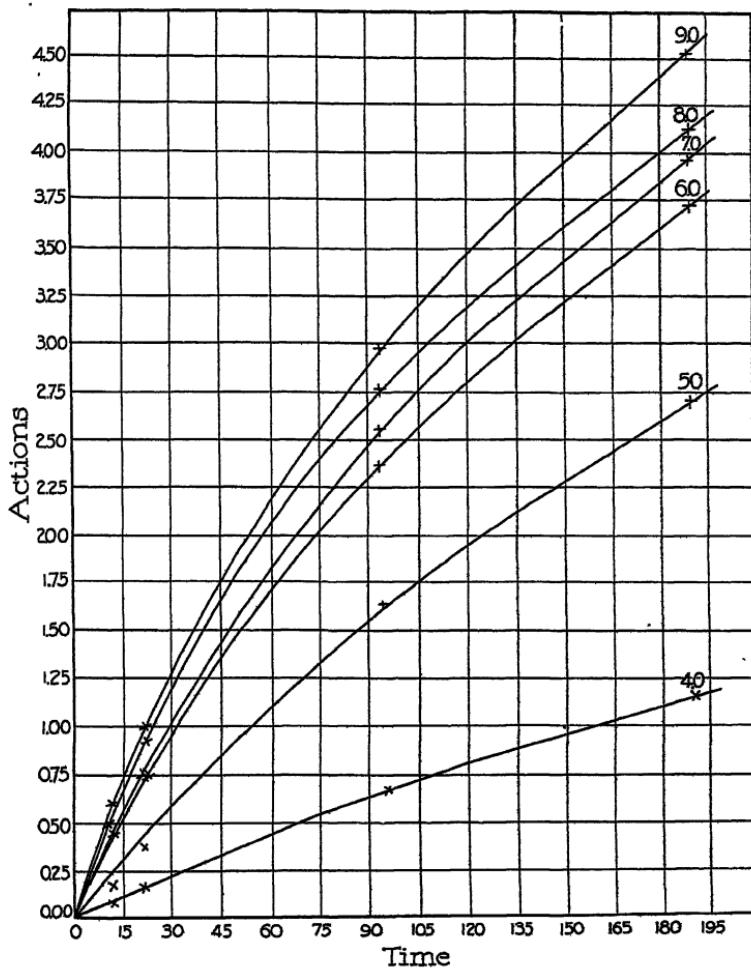


FIG. 3. Time-action results of rat tumor extract, No. 49, on glyceryl triacetate, initially at the different pH values indicated. Abscissa values,  $\frac{1}{2}$  hour units; ordinate values, tenths of milli-equivalents of ester hydrolyzed.

Table X. The more complete tumor extract series shows a remarkable constancy at the successive time intervals when it is considered that the actions were started at different hydrogen ion concentrations but had all reached the same constant value in

less than 3 hours. If there had been no adverse action at the different initial hydrogen ion concentrations, the actions after 3 hours should have been alike. The results indicate that the different conditions of acidity inactivated different amounts of enzyme, the most alkaline solution the least, but that after the initial inactivation, the activities of the various mixtures remained unchanged or were affected to the same degree during

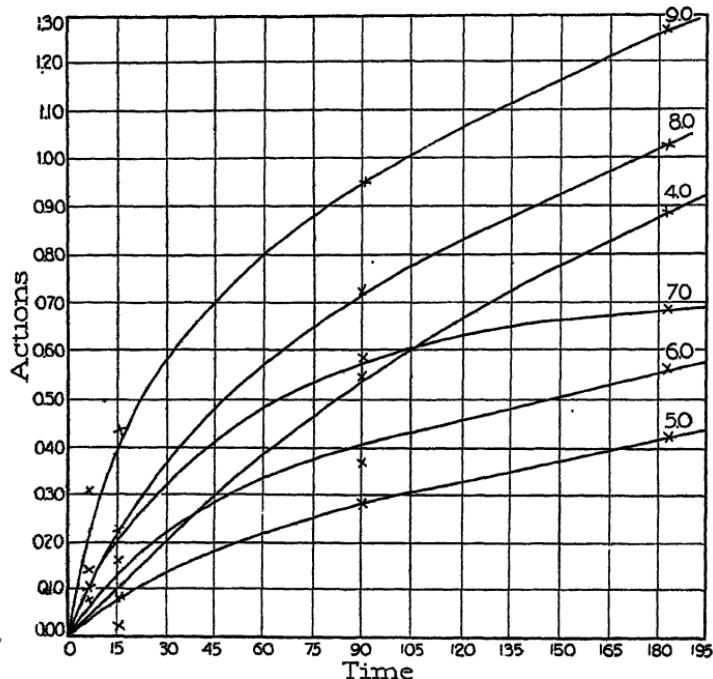


FIG. 4. Time-action results of rat leg muscle extract, No. 50, on glyceryl triacetate, initially at the different pH values indicated. Abscissa values,  $\frac{1}{4}$  hour units; ordinate values, tenths of milli-equivalents of ester hydrolyzed.

the rest of the experiment. It was shown in the former paper that the rate of inactivation was least at pH 7.0. In the experiments just described, the mixtures did not remain at the initial hydrogen ion concentrations long enough to show this difference in the rate of inactivation; the greater activity in the most alkaline solution being the predominating action observable.

The results obtained in three experiments, two with glyceryl acetate and one with phenyl acetate, in which different amounts enzyme and substrate were used are given in Table XI. The

TABLE VIII.

*Ions, in Tenth of Milli-Equivalents of Ester Hydrolyzed, of Tumor and Muscle Tissue Extracts on Glyceryl Triacetate Starting at Different Hydrogen Ion Concentrations.*

Times actions in hr. units.	Initial pH of mixtures.					
	4.0	5.0	6.0	7.0	8.0	9.0
Experiment 49. Tumor extract.						
11½	0.06	0.15	0.43	0.50	0.50	0.59
21½	0.16	0.35	0.73	0.74	0.92	0.99
95½	0.66	1.63	2.37	2.56	2.76	2.97
90	1.14	2.69	3.71	3.96	4.10	4.51
Experiment 50. Muscle extract.						
7	0.08	0.14	0.10	0.10(6.6)	0.14(6.8)	0.31(7.0)
16	0.02	0.08	0.16	0.22(5.6)	0.20(5.8)	0.43(6.4)
91	0.54	0.28	0.36	0.58	0.72	0.95
84	0.88	0.42	0.56	0.68	1.02	1.27

TABLE IX.

*Ions Required for the Same Actions Starting at Different Hydrogen Ion Concentrations from the Results of Table VIII and Figs. 3 and 4.*

Actions in hr. units.	Times ( $\frac{1}{10}$ hr. units) for indicated actions with initial pH values.					
	4.0	5.0	6.0	7.0	8.0	9.0
Experiment 49. Tumor extract.						
1.00	161	54	31	30	24	22
2.00		126	75	69	58	54
3.00		222	136	122	110	97
4.00			214	193	182	155
Experiment 50. Muscle extract.						
0.50	(82)	240	150	64	48	22
1.00	(219)				177	104

tions were allowed to proceed for 22 hours. The relative enzyme concentration in any one series is given under *E*, the initial substrate concentration, *A*, in tenths of milli-equivalents, and the

TABLE X.

*Relative Enzyme Contents Calculated Assuming the Constancy of the Product ET from the Results of Table IX for Successive Amounts of Action.*

Successive amounts of action for which times ( $T$ ) were used.	Calculated relative enzyme contents for successive amounts of actions for mixtures initially at indicated pH.					
	4.0	5.0	6.0	7.0	8.0	9.0
Experiment 49. Tumor extract.						
0.00-1.00	14	41	71	(73)	92	100
1.00-2.00		44	73	82	94	100
2.00-3.00		45	70	81	83	100
3.00-4.00			74	82	81	100
Experiment 50. Muscle extract.						
0.00-0.50		9	15	34	46	100
0.50-1.00					64	100

TABLE XI.

*Actions in Terms of Tenths of Milli-Equivalents of Ester Hydrolyzed with Varying Concentrations of Enzyme and Substrate.*

Experiment No.	Ester.	Relative enzyme concentration, $E$ .	Milli-equivalents of ester, $A$ .			
			6.8	20.4	34.0	68.0
45	Glyceryl tri-acetate.	1	0.09	0.15	0.13	0.15
		2	0.17	0.29	0.31	0.36
		4	0.37	0.60	0.62	0.72
		6	0.55	0.81	0.81	1.01
		8	0.72	1.04	1.10	1.27
46	Glyceryl tri-acetate.	1	0.24	0.38	0.44	0.53
		2	0.36	0.65	0.78	0.96
		4	0.66	1.22	1.38	1.72
		6	1.13	1.60	1.87	2.31
		8	1.22	2.10	2.37	2.80
			7.9	23.7	39.5	79.1
59	Phenyl acetate.	1	1.27	1.42	1.53	1.55
		2	2.12	2.60	2.67	2.74
		4	2.96	4.39	4.81	4.80
		6	3.95	5.61	6.27	6.48
		8	4.36	6.87	7.53	8.18

actions,  $x$ , in the body of the table in terms of tenths of milliequivalents of acid formed by the actions, corrected for blanks. The mixtures were brought to pH 7.0 initially, but fell rapidly to about pH 5.2 to 5.3 at which point they remained practically constant. The contents in terms of enzyme material extracted were as follows:

- Experiment 45.....7.5, 15.0, 30.0, 45.0, and 60.0 mg. of tumor tissue per cc. of mixture tested.
- Experiment 46.....34.4, 68.8, 137.6, 206.4, and 275.2 mg. of tumor tissue per cc. of mixture tested.
- Experiment 59.....6.8, 13.6, 27.1, 40.7, and 54.3 mg. of tumor tissue per cc. of mixture tested.

While some irregularities appear in the results due to experimental errors, the amounts of the actions increase in every case with increasing enzyme concentration. With any one enzyme concentration, however, in a number of the series the actions appeared to have approached or reached a maximum. The only simple relation evident is that in Experiment 45; with any one ester concentration the actions are proportional to the enzyme concentrations.

In order to attempt a formulation of the results, the values of  $K$  of Schütz's and Northrop's equations were calculated. A similar calculation using Northrop's equation was not possible for the protease actions of tumor and tissue extracts reported previously, because the total actions were not known. In the present instance this total action is simply placed equal to the initial substrate concentration,  $A$ .

The values given in Table XII were calculated from Schütz's equation  $K_s = \frac{x}{\sqrt{EA}}$ ; those in Table XIII from Northrop's equation  $K_n = \frac{A}{E} \log_e \frac{A}{A-x} - \frac{x}{E}$ ;  $T$  being constant.

The values of  $K_s$  and  $K_n$  in Tables XII and XIII show no indication of constancy. It is possible that part of this irregularity is due to the change in pH from 7.0 to about 5.2 during the first part of the reaction. The regularities apparent include, with few exceptions, steady decreases with increasing substrate concentrations for a given enzyme concentration, and steady increases with increasing enzyme concentrations for a given substrate concentration.

TABLE XII.

*Values of  $K_s \times 10^2$  Calculated from Schütz's Equation from the Results of Table XI.*

Experiment No.	Relative enzyme concentration, E.	Substrate concentrations, A.			
		6.8	20.4	34.0	68.0
45	1	3.5	3.3	2.2	1.8
	2	4.6	4.6	3.8	3.1
	4	7.2	6.6	(3.3)	4.4
	6	8.6	7.3	5.7	5.0
	8	9.8	8.1	6.7	5.4
46	1	9.2	8.4	7.6	6.4
	2	9.8	10.2	9.5	8.3
	4	12.6	13.5	11.8	10.4
	6	17.8	14.5	13.1	11.4
	8	16.5	16.4	14.4	12.0
		7.9	23.7	39.5	79.1
59	1	45	29	24	17
	2	54	38	30	22
	4	50	45	38	27
	6	58	51	41	30
	8	55	50	42	33

TABLE XIII.

*Values of  $K_n \times 10^3$  Calculated from Northrop's Equation from the Results of Table XI.*

Experiment No.	Relative enzyme concentration, E.	Substrate concentrations, A.			
		6.8	20.4	34.0	68.0
45	1	0.58	0.55	0.22	-0.40
	2	1.09	1.06	0.89	0.54
	4	2.3	2.2	1.5	0.88
	6	3.6	2.8	1.8	0.75
	8	5.1	3.4	2.2	1.5
46	1	4.2	3.7	3.0	1.8
	2	4.9	5.3	4.7	3.1
	4	8.6	9.5	7.2	5.5
	6	17.5	11.0	8.9	6.6
	8	14.8	13.7	10.8	7.4
		7.9	23.7	39.5	79.1
59	1	114	44	30	15
	2	174	80	47	24
	4	187	116	79	38
	6	254	131	93	47
	8	247	155	102	56

A number of experiments carried out with some further animal tissues and with tumors of human origin will not be given here. These series were less complete than those already given. The general relations were similar, but added nothing to the conclusions based on the more complete series of experiments.

#### DISCUSSION.

The results given in this paper show that the kinetic relations of the lipolytic actions of the extracts used were similar to those of other enzyme actions. No completely satisfactory mathematical theory of such actions has been developed as yet. The equations which have been developed by others and which are used here apply in certain more or less extended ranges of the experiments, but no one view has been found to be of general applicability. It is extremely probable that enzyme actions take place in steps, that frequently the products of the reaction interfere with the actions, that a change in hydrogen ion concentration during the course of the actions complicates the interpretation, etc. The deviations from the theoretical equations which have been deduced in the past have in certain cases been satisfactorily explained on rational chemical grounds. Such explanations have been used where possible in discussing the experimental results given in the preceding section. There is no necessity for describing in detail or for elaborating the theoretical views which have been used here, since nothing has been added to such theoretical treatments.

#### SUMMARY.

The kinetics of the hydrolytic actions on glyceryl triacetate of extracts of the Flexner-Jobling rat carcinoma and of rat leg muscle were studied and interpreted on the basis of the monomolecular reaction velocity equation, of Schütz's equation, and of Northrop's equation. The general relations found were similar to those found by others and are probably explainable on analogous grounds.

A study of the hydrolytic actions on esters with different enzyme and substrate concentrations showed that none of the theoretical equations was generally applicable, but that in certain series comparatively simple relations held within limits.

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